Targeting muscarinic receptor subtypes as a therapeutic approach in dystonia and Parkinson's disease

Klisko, Dominika Maria

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Targeting muscarinic receptor subtypes as a therapeutic approach in dystonia and Parkinson's disease

Dominika Maria Klisko

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Certificate

This is to certify that the research work embodied in this thesis has been carried out by me under the supervision and guidance of Dr Sarah Salvage and Professor Peter Jenner.

Dominika Klisko

Abstract

Anticholinergics show therapeutic utility in the treatment of some types of dystonia and in Parkinson's disease, however, this is accompanied by wide range of unpleasant side effects, including dry-mouth. It has been proposed that muscarinic M4 receptors, located in the striatum, could be a novel therapeutic target to minimise involuntary movements and improve parkinsonian disability without producing unfavourable peripheral side effects.

The effect of muscarinic antagonists with different relative selectivity for receptor subtypes, including a novel highly selective muscarinic antagonist for the M4 subtype, have been investigated using the pilocarpine-induced purposeless chewing model of dystonia in rats and MPTP-treated common marmoset model of PD. The peripheral effects of these drugs were assessed on pilocarpine-induced saliva secretion in rats.

Pilocarpine purposeless chewing was significantly inhibited by centrally but not peripherally acting anticholinergics including the novel M4 selective antimuscarinic. It was confirmed that M1/M3 selective antimuscarinics are important in the onset of dry-mouth, and that this effect was peripherally mediated. Moderate suppression of salivation was also observed with M1 and M2 antimuscarinics, but not with the selective M4 antagonist. This suggests that selective blockade of muscarinic M4 receptors may have a role in dystonia without accompanying peripheral side effects.

Interestingly, in MPTP-treated marmosets, co-administration of clinically used centrally acting anticholinergics alone and in combination with L-DOPA resulted in the expected improvement of locomotor activity and motor disability but also enhanced expression of L-DOPA-induced dyskinesia. By contrast, selective muscarinic M4 antagonist showed no significant improvement in locomotion and motor disability either alone or in combination with L-DOPA and did not reduce the expression of L-DOPA-induced dyskinesia, however, extended duration of dystonia.

Overall, these studies support the idea that cholinergic system plays a role in mediation of motor control, and that selective antagonism of M4 receptors may reduce dystonia without inducing peripheral side effects, however, the lack of effect on motor function and the increase in drug-induced dystonia in the Parkinson's disease model suggest no position in this disease.

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3-OMD	3-Methyl-tyrosine hydrate
5-HIAA	5-Hydroxyindole-3-acetic acid
5-HT	5-Hydroxytryptamine (Serotonin)
6-OHDA	6-hydroxydopamine
ACh	Acetylcholine
AMD	α-Methyldopa
ANOVA	Analysis of variance
AUC	Area under the curve
BBB	Blood brain barrier
BG	Basal ganglia
CNS	Central nervous system
CO_2	Carbon dioxide
DA	Dopamine
DAT	Dopamine transporter
DHBA	3,4-Dihydroxybenzylamine hydrobromide
DOPAC	3,4-Dihydroxypenylacetic acid
ED_{50}	Median effective dose
EDTA	Ethylenediaminetetraacetic acid
GABA	Gamma-amino butyric acid
GI	Gastrointestinal
GLU	Glutamate
GP	Globus pallidus
HCl	Hydrochloride, hydrochloric acid
HPLC	High-performance liquid chromatography
HVA	Homovanillic acid
ICV	Intracerebroventricular

ID_{50}	Half maximal inhibitory dose
IP	Intraperitoneal
L-DOPA	L-3,4-dihydroxyphenylalanine
LID	L-DOPA-induced dyskinesia
LTD	Long term depression
LTP	Long term potentiation
LV	Lateral ventricle
MAO	Monoamine oxidase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
OSA	Octane-1 sulphonic acid
PBS	Phosphate buffered saline
PCA	Perchloric acid
PD	Parkinson's disease
PNS	Peripheral nervous system
PO	*Per os* (oral administration)
SC	Subcutaneous
SEM	Standard error of the mean
SN	Substantia nigra
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
VTA	Ventral tegmental area

Publications

Posters

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Chapter 1 General Introduction

1.1 Movement disorders

Movement disorders are neurologic conditions resulting from problems with the control of the "quality" of the movement – either an excess or lack of voluntary and automatic movements (Fahn, 2011). They can arise from malfunctioning of the muscles or abnormality in motor pathways in the nervous system. In particular, dysfunction and pathological processes in the basal ganglia and their connections are associated with a number of movement disorders.

Movement disorders can be divided into two different categories: hyperkinetic such as dystonia, dyskinesia, chorea, tremor, where movements are involuntary and repetitive, often interfering with normal motor activity, and hypokinetic, such as Parkinson's disease (PD), where there is impairment of voluntary movement such that movement is reduced or absent (Fahn, 2011). There are many and varied forms of treatment for these disorders, however, anticholinergic drugs are used both for some dystonias and in PD. These drugs are effective, but are also associated with unpleasant side effects that reduce compliance, therefore, improved anticholinergic therapy is required for both these disorders. Thus, the studies in this thesis describe preclinical studies that aim to investigate improved anticholinergic therapy for dystonia and PD.

1.1.1 Dystonia

Dystonia is a neurological hyperkinetic movement disorder characterised by extreme, sustained and involuntary muscle contraction. It was firstly described in 1908 by a German physician Markus Walter Schwalbe in his doctoral thesis as he observed it in children of Jewish origin as a syndrome of tonic cramps (Truong & Fahn, 1988; Grundmann, 2005). However, three years later, a German neurologist Hermann Oppenheim termed this condition as dystonia musculorum deforemans based on observation of children affected by unusual uncontrolled muscle spasms, leading to twisting, rapid and rhythmic movements which were worsening on walking and eventually leading to abnormal and fixed postures (Klein & Fahn, 2013). This condition was later referred to as primary torsion dystonia (Grundmann, 2005). For many decades dystonia has been considered as a psychogenic disorder, however, work and observations made by C. David Marsden in 1975 on adult-onset dystonia have changed the perception and dystonia started to be considered as a neurological hyperkinetic movement disorder (Marsden, 1976; Skogseid, 2014). Years later in 1984

a scientific committee of the Dystonia Medical Research Foundation provided the first definition of dystonia, which stated that it is a syndrome of sustained muscle contractions, causing twisting and repetitive movements, or abnormal postures (Skogseid, 2014). This description has been widely used for years until the international committee of movement disorders and experts in dystonia proposed a new revised definition:

"*Dystonia is a movement disorder characterised by sustained or intermittent muscle contractions causing abnormal, often repetitive, movements, postures, or both. Dystonic movements are typically patterned, twisting, and may be tremulous. Dystonia is often initiated or worsened by involuntary action and associated with overflow muscle activation*" (Albanese *et al.*, 2013).

1.1.1.1 Clinical features

In dystonia, primary muscles normally responsible for a movement contract excessively together with the nearby muscles that generally do not take part in the movement. Hence, the dystonic symptoms are triggered by involuntary simultaneous contractions of agonist and antagonist muscles, resulting in repetitive and patterned movements. These movements can be rapid or slow and can change depending on activities or postures. The abnormal movement depends on the strength and combination of muscles involved in a particular movement. Mild dystonia results in exaggeration of normal movements; moderate cases comprise of stiff, slow, twisting or jerky movements; and severe forms lead to unnatural postures and fixed deformities including the trunk, neck, face and/or limbs. Generally, these sustained muscle spasms are painful, and tend to become worse during action, and improve during relaxation. Some dystonic movements occur only during voluntary activities (task-specific or action dystonia), others are triggered by sensory tricks, such as gently touching or poking the affected body part (geste antagoniste) (de Carvalho Aguiar & Ozelius, 2002; Tarsy & Simon, 2006; Cloud & Jinnah, 2010). Dystonia can involve muscle groups throughout the entire body (generalised), or can be specific to a certain area of the body (focal) (Figure 1.1) (Jinnah & Hess, 2008).

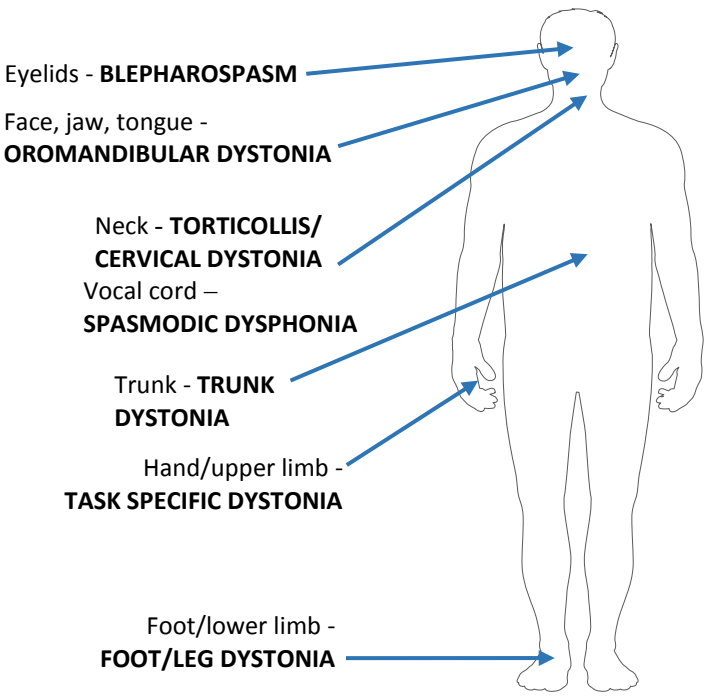


Figure 1.1 Example of dystonia and body region affected.

1.1.1.2 Epidemiology

Dystonia affects many people worldwide, regardless the age or gender, and is considered as a third most common movement disorder, after Parkinson's disease and tremor disorders (Defazio *et al.*, 2004; Langlois *et al.*, 2014). Worldwide more than 3 million people are diagnosed with dystonia (Jinnah *et al.*, 2008). According to The Dystonia Society UK, there are around 70,000 people affected by dystonia in the UK (of these 8,000 children), resulting in prevalence of 1 in 900 (The Dystonia Society, 2016).

Despite dystonia being one of the most prevalent movement disorder, the epidemiological investigation is limited. Diverse aetiologies and different manifestations of disease make it difficult to diagnose. Additionally, dystonia is still considered as a rare condition, and since it is non-fatal, there is a lack of epidemiological studies (Jinnah *et al.*, 2008; Langlois *et al.*, 2014). A study conducted in eight European countries estimates that 15.2 per 100,000 suffers from primary dystonia, with the highest level for focal dystonia (11.7 per 100,000) (The Epidemiological Study of Dystonia in Europe (ESDE) Collaborative Group, 2000).

Based on this report, there is a difference in prevalence and age at onset in terms of gender, as women tend to be more susceptible to focal dystonia, and tend to have higher risk and lower age at onset for writer's cramp, whereas men have lower age at onset for blepharospasm, cervical and laryngeal dystonia (The Epidemiological Study of Dystonia in Europe (ESDE) Collaborative Group, 2000; Defazio *et al.,* 2004).
In addition, a systematic review and meta-analysis of numerous studies on prevalence, incidence and epidemiology of dystonia reports that the prevalence of primary dystonia was 16.4 per 100,000 (Steeves *et al.*, 2012).

1.1.1.3 Classification and aetiology

In recent years, the revised classification of dystonia has emerged and is based on two axes: clinical characteristics and aetiology (Albanese *et al.,* 2013).
The clinical characteristics important in classification of dystonia are: age at onset, body distribution, temporal pattern, coexistence of other movement disorders and coexistence of other neurological manifestations (Table 1.1).
Dystonia can develop at any age, and is defined as infancy (0-2 years), childhood (3-12 years), adolescence (13-20 years), early adulthood (21-40 years) and late adulthood (>40 years) onset. In the early adulthood onset (age <20-40 years), dystonia typically begins as a focal form (limbs, usually the leg) and tend to progress to other body areas and develops into severe generalized dystonia in majority of the patients. Late adulthood-onset (age >40 years) begins in the neck, face or arm and is not usually progressive, thus remains focal or segmental (de Carvalho Aguiar & Ozelius, 2002; Bhidayasiri, 2006; Tarsy & Simon, 2006; Jinnah *et al.,* 2008). In dystonia, different body parts can be involved individually or in combinations, the classification in terms of body distribution is divided into focal, segmental, multifocal, generalized and hemidystonia (Table 1.1). The body distribution may change over time and can progress to the body parts that were not previously involved (Jinnah *et al.,* 2008; Albanese *et al.,* 2013).
Classification in terms of temporal pattern distinguishes between static or progressive disease and could include four different patterns: persistent, action-specific, diurnal fluctuations or paroxysmal.

Table 1.1 Classification of dystonia by clinical characteristics and aetiology (Albanese *et al.*, 2013).

Clinical characteristics	
Age of onset	• Infancy: birth to 2 years • Childhood: 3 to 12 years • Adolescence: 13 to 20 years • Early adulthood: 21 to 40 years • Late adulthood: >40 years
Body distribution	• Focal: involving a single body region • Segmental: two or more contiguous body regions • Multifocal: two non-contiguous or more body regions • Generalized: the trunk and at least two other sites • Hemidystonia: more regions restricted to one body side
Temporal pattern	• Disease course: static or progressive • Variability: - Persistent: approximately same extend throughout the day - Action-specific: occurs only during a specific activity or task - Diurnal: fluctuating dystonia during the day, with identifiable circadian variations in occurrence, severity and phenomenology - Paroxysmal: unexpected self-limited episodes of dystonia often induced by a trigger with return to pre-existing neurologic state
Associated features	• Isolated or combined with another movement disorder: - Isolated: dystonia is the only motor feature (with the exclusion of tremor) - Combined: dystonia is combined with other movement disorder (myoclonus, parkinsonism) • Occurrence of other neurologic or systemic manifestations
Aetiology	
Nervous system pathology	• Evidence of degeneration (e.g. neuronal loss) • Evidence of structural lesions • No evidence of degeneration and structural lesions
Inherited or acquired	• Inherited (proven genetic origin): - Autosomal dominant - Autosomal recessive - X-linked recessive - Mitochondrial • Acquired (due to a known specific cause): - Cerebrovascular (infarction or haemorrhage) - Perinatal brain injury (delayed-onset dystonia, dystonic cerebral palsy) - Traumatic brain injury (brain surgery, electrical injury) - Infection (syphilis, tuberculosis, HIV, viral encephalitis) - Drug (anticolvusants, calcium channel blockers, dopamine agonists, neuroleptics/antiemetics) - Toxic (carbon disulphide, cobalt, manganese, methanol) - Neoplastic (brain tumour, paraneoplastic encephalitis) - Psychogenic
Idiopathic (unknown cause)	• Sporadic • Familial

Dystonia can often occur in isolation, which means that it is the only motor disability, except the presence of tremor, or can be combined with another movement disorder, such as myoclonus or parkinsonism. In addition, other neurological or systemic manifestations can be present along with dystonia (Table 1.1) (Albanese *et al.*, 2013).

Aetiology of many forms of dystonia is still poorly understood. The classification in respect of aetiology involves presence or absence of nervous system pathology, which may involve evidence of degeneration or structural lesions, and the division between inherited, acquired and idiopathic forms (Albanese *et al.*, 2013).

Cases of inherited dystonia have a proven genetic origin and different forms can be distinguished: autosomal dominant, autosomal recessive, X-linked recessive or mitochondrial (Table 1.2) (Albanese *et al.*, 2013).

Acquired dystonia, previously known as secondary dystonia, usually arises due to a known specific underlying cause or condition and this may include perinatal brain injuries (dystonic cerebral palsy, delayed-onset dystonia), infections (including viral encephalitis, encephalitis lethargics, human immunodeficiency virus (HIV) infection, tuberculosis, syphilis), drugs (e.g. levodopa and dopamine agonists, neuroleptics, anticonvulsants, calcium channel blockers), toxic (manganese, cobalt, cyanide, carbon disulphide, methanol, 3-nitropropionic acid, disulfiram), vascular (ischaemia, haemorrhage, arteriovenous malformation), neoplastic disease (brain tumour, paraneoplastic encephalitis), brain injuries (head trauma, brain surgery, electrical injury),or psychogenic causes (functional). Idiopathic cases, so with unknown cause, can be divided into sporadic and familial (Albanese *et al.*, 2013).

Table 1.2 Genes underlying dystonia.
AD – autosomal dominant; AR – autosomal recessive; ATP – adenosine triphosphate. Adapted and modified from Warner & Jarman (1998); Charlesworth *et al.* (2013b); Lohmann & Klein (2013); Waugh & Sharma (2013); Klein (2014).

Gene Locus	Designation	Mode of inheritance	Chromosome, mutation and gene product
DYT1	Dystonia 1, torsion	AD	9q34; GAG deletion in TOR1A gene causes abnormality in ATP-binding protein, torsinA
DYT2	Dystonia 2, torsion	AR	Unknown chromosome, gene product
DYT3	X-linked dystonia-parkinsonism (Lubag)	X-linked recessive	Xq13.1; TAF1 gene

DYT4	Whispering dysphonia	AD	19p13.3; mutations in the TUBB4A gene
DYT5	Dopa-responsive dystonia/parkinsonism	AD	14q22; GCH1 locus; mutations in GTP cyclohydrolase I gene
	Segawa syndrome	AR	1p15.5; mutations in the tyrosine hydroxylase gene; tyrosine hydroxylase deficiency
DYT6	Dystonia 6, torsion	AD	8p11.21; mutations in the THAP1 gene
DYT7	Dystonia 7, torsion	AD	18p; gene and product unknown
DYT8	Paroxysmal nonkinesigenic dyskinesia 1	AD	2q35; myofibrillogenesis regulator (MR1 gene)
DYT9 (allelic with DYT18)	Episodic kinesigenic dyskinesia 1 (paroxysmal kinesigenic dyskinesia)	AD	1p34.2; mutations in the SLC2A1 gene that encodes the glucose transporter type 1
DYT10	Episodic kinesigenic dyskinesia 1 (paroxysmal kinesigenic dyskinesia)	AD	16p11.2; mutations in the PRRT2 gene
DYT11	Myoclonus-dystonia	AD	7q; epsilon-sarcoglycan gene (SGCE)
DYT12	Rapid onset dystonia-parkinsonism	AD	19q; ATP1A3 gene that encodes the Na+/K+ -ATPase alpha3 subunit
DYT13	Dystonia 13, torsion	AD	1p; gene and product unknown
DYT14	Dopa responsive dystonia (now included under DYT5)	Not clear	Unknown
DYT15	Dystonia 15, myoclonic	AD	18p11; gene and product unknown
DYT16	Dystonia 16	AR	2q31.3; PRKRA gene
DYT17	Dystonia 17, torsion	AR	20p11.2-q13.12; gene and product unknown
DYT18 (allelic with DYT9)	Paroxysmal exercise-induced dyskinesia with or without epilepsy and/or hemolytic anemia (GLUT1 deficiency syndrome 2)	AD	1p34.2; mutations in the SLC2A1 gene that encodes the glucose transporter type 1
DYT19	Episodic kinesigenic dyskinesia 2 (paroxysmal kinesigenic dyskinesia)	AD	16q13-q22.1; gene and product unknown
DYT20	Paroxysmal nonkinesigenic dyskinesia 2	AD	2q31; gene and product unknown

DYT21	Dystonia 21	AD	2q14.3-q21.3; gene and product unknown
DYT23	Dystonia 23	AD	9q34, ?CIZ1 gene
DYT24	Dystonia 24	AD	11p14.2, ANO3 gene
DYT25	Dystonia 25	AD	18p11.21, GNAL gene

1.1.1.4 Pathogenesis

Pathophysiology of dystonia is complex and the absence of cell degeneration proposes that this disorder comes from abnormal cell function. Dysfunction of basal ganglia, however, plays an enormous role in dystonia, but the specific mechanisms are unclear. Lesions within basal ganglia structures, including caudate, putamen, globus pallidus and thalamus were found in dystonic patients (Marsden *et al.*, 1985). Surgical approaches, which lesion the GPi or STN, proved to be effective in some dystonias (Krauss *et al.*, 2004; Toda *et al.*, 2004). There is an underlying genetic component linked to many forms of dystonia and several genes and loci have been identified to the dystonia DYT loci and genes (Table 1.3) (Charlesworth *et al.*, 2013a). Additionally, further evidence from neuroimaging studies, i.e. positron emission tomography (PET) fMRI show that dystonia can arise not only form abnormal activity in the basal ganglia or motor cortex but also from the cerebellum, therefore dysfunction of other brain regions or networks also contribute to this disorder, thus dystonia can be seen as neurodevelopmental circuit disorder and may involve the cortico-striato-pallido-thalamo-cortical and cerebello-thalamo-cortical pathways (Galardi *et al.*, 1996; Eidelberg *et al.*, 1998; Argyelan *et al.*, 2009; Neychev *et al.*, 2011; Niethammer *et al.*, 2011). Imaging studies show abnormal cerebellar function in patient carrying DYT1 and DYT6 mutations, most commonly inherited forms of dystonia (Eidelberg *et al.*, 1998; Argyelan *et al.*, 2009). Furthermore, the lesions and tumours of the cerebellum have been found in patients with cervical form of dystonia (LeDoux & Brady, 2003; Prudente *et al.*, 2014). Several animal models such as dt^{sz} hamster or 3-nitropropionic acid-induced rat model are associated with basal ganglia dysfunction, but there are also models associated with cerebellar involvement in the disorder, such as dystonic (dt) rat model, DYT1 mutant mice with the mutation of torsin1A gene or tottering mutant mouse, and toxin-induced mice or rat models of kainic acid

microinjection (LeDoux *et al.*, 1998; Pizoli *et al.*, 2002; Jinnah *et al.*, 2005; Raike *et al.*, 2005; Jinnah *et al.*, 2008; Neychev *et al.*, 2008; Alvarez-Fischer *et al.*, 2012).

Findings from electrophysiological studies, including transcranial magnetic stimulation (TMS), show a loss of inhibition at various level of the motor system in dystonia. This can be related to some manifestation of dystonia, such as prolonged muscle contractions, involuntary co-contractions of groups of muscles seen, for example, in blepharospasm, where there is an abnormality in recovery in the blink reflex (Berardelli *et al.*, 1998; Hallett *et al.*, 2006). Nevertheless, loss of inhibition cannot be always the case as these abnormalities can also be found in carriers of DYT1 gene mutation without clinical manifestation (Edwards *et al.*, 2006).

Abnormal neuronal signalling within the basal ganglia and abnormal synaptic plasticity may contribute to the disease (Martella *et al.*, 2009). Paired associative stimulation (PAS) and TMS are commonly used techniques to study plasticity at the cortical level. The long-term potentiation (LTP) and long-term depression (LTD) are recognised as models of plasticity (Bonsi *et al.*, 2008). Patients with focal dystonia show increased response to different experimental plasticity protocols (Quartarone *et al.*, 2003; Edwards *et al.*, 2006; Weise *et al.*, 2006). The abnormalities were observed in hand muscles of patients with focal dystonia affecting other body region who had no visible symptoms in hand muscles, suggesting a generalized type of the disease (Quartarone *et al.*, 2008). Repetitive activity or performance of a particular movement for a long period of time could lead to dystonic symptoms, for instance in focal limb dystonia. This has been shown using primates when overtraining in specific hand movements made the animals unable to continue. Observed motor abnormality was interpreted as dystonia (Byl *et al.*, 1996). Further studies examining somatosensory cortex showed that the receptive field was enlarged and overtraining led to abnormal sensory function causing abnormalities in motor function (Byl *et al.*, 1996). Therefore, abnormality in sensorimotor cortex may contribute to pathophysiology of dystonia, triggering changes in plasticity, such as performance of repetitive movements (Quartarone & Pisani, 2011).

Neurochemically, it has been suggested that both the dopaminergic and cholinergic systems are involved in the manifestation of dystonia. Imaging studies have demonstrated reduced D2 receptor binding in the striatum in patients with DYT1 and

DYT6 dystonia (Beukers *et al.*, 2008; Carbon *et al.*, 2010; Karimi *et al.*, 2011). Both anticholinergic and dopaminergic pharmacological treatments are effective in some dystonias.

1.1.1.5 Dystonia Therapy

Notwithstanding the advances made in the understanding of the pathophysiology of dystonia, the underlying cause remains unknown and to date there is still no cure. The treatment of many forms of dystonia is generally symptomatic and consequently the primary aim is to decrease abnormal movements, correct and improve posture, reduce pain and generally improve quality of life of sufferers (Bhidayasiri, 2006; Jankovic, 2006; Langlois *et al.*, 2014). Treatment of dystonia is problematic due to many aetiologies and heterogeneous clinical appearances involving different parts of the body (Jankovic, 2013), therefore, many patients require combination of different forms of treatment (Jankovic, 2009a). For many years, the treatment was dependent on the use of pharmacological agents, which shows moderate effects but also their use is related to many side effects (see below). The treatment has changed in the past years, as other therapeutic options have been introduced, such as botulinum toxin or surgery, including peripheral denervation, pallidotomy and thalatomy or deep brain stimulation (DBS), with the latter the most widely currently used surgical procedure (Cloud & Jinnah, 2010; Lubarr & Bressman, 2011). Patients with focal form (late-onset) are given local injections of botulinum toxin in the affected muscles (Bhidayasiri, 2006; Jankovic, 2006; Langlois *et al.*, 2014). Deep brain stimulation of the globus pallidus internus (GPi) is used in severe cases of early-onset primary generalized dystonia or sometimes in patients suffering from focal dystonia (cranial and cranial-cervical forms) (Jankovic, 2009b; Lubarr & Bressman, 2011). The downside is that DBS is only offered to treat severe cases of dystonia and is not widely available, as it has to be conducted by the team of specialists involving neurologists, neurosurgeons, psychiatrists and neuropsychologists (Jinnah & Hess, 2008; Lubarr & Bressman, 2011).

Pharmacological treatment is also tailored to individual patients and type of syndrome they experience. Patients with focal, segmental and generalized dystonia are mainly treated with oral medications, including anticholinergics (trihexyphenidyl), the dopamine precursor - levodopa, dopamine antagonists (phenothiazines, tetrabenazine)

and the $GABA_b$ agonist (baclofen). Other pharmacological medications, such as muscle relaxants, benzodiazepines (diazepam, lorazepam, clonazepam) or anticonvulsants may also provide benefit in improvement of some types of dystonia (Goldman & Comella, 2003).

Physiotherapy is an important and useful addition to any treatment of dystonia. Its main aim is to improve posture, increase strength and mobility of a patient and work to prevent the contractures in patients suffering from generalised dystonia (Goldman & Comella, 2003; Lubarr & Bressman, 2011; Thenganatt & Jankovic, 2013).

Despite the fact that there are several forms of treatment available, the broad range of manifestations and different causes of dystonia, they are not always effective and, therefore, there is an unmet need for developing more effective forms of therapy, or improve existing ones, and this is still the subject of further investigation.

1.1.1.5.1 Pharmacological treatment

Oral medications are mainly used in segmental and generalised dystonia and as an adjunct therapy to the botulinum toxin in focal dystonia. Treatment is usually limited due to many side effects, however, children often better tolerate much higher doses than adults with good benefit. In any case, the treatment should be slowly titrated over period of time to reduce the side effects (Thenganatt & Jankovic, 2013). Pharmacological treatment of dystonia is mostly based on empirical rationale rather than on data coming from clinical trials or scientific knowledge (Jankovic, 2009a). Among the pharmacological forms of treatment few classes of oral medications have demonstrated to be effective in reducing the symptoms of dystonia.

GABA agonists

Baclofen, a presynaptic $GABA_B$ receptor agonist, mimics the major inhibitory neurotransmitter gaba-aminobutyric acid (GABA) in the brain. By activation of $GABA_B$ sites, which are present on nerve terminals, $GABA_B$ agonists reduce neurotransmitter release by inhibition of calcium influx, which is associated with inhibition of motor neurons in spinal cord and thus preventing muscle contractions (Greene, 1992). Treatment with baclofen may be helpful in children with dystonic gait and patients suffering from segmental, generalized or oromandibular dystonia, as well as in Parkinson's disease for wearing-off or early-morning foot dystonia (Greene,

1992; Jankovic, 2013). Despite that, the treatment with baclofen results in number of side effects, including drowsiness, dizziness, nausea, constipation and dry mouth (Goldman & Comella, 2003; Cloud & Jinnah, 2010; Lubarr & Bressman, 2011).

Dopaminergic agents

Dopaminergic agents can control movement by acting on the nigro-striatal dopamine system, which helps control muscle movement. Levodopa, the precursor of dopamine, is converted to dopamine in DA neurons and is used in the management of dopa-responsive dystonia (DRD), a syndrome caused by a defect in synthesis of dopamine, particularly in childhood-onset dystonia (Cloud & Jinnah, 2010; Thenganatt & Jankovic, 2013). Studies show that in general patients respond well to low doses of levodopa (300 mg/day), however, many patients need higher doses (up to 1000 mg/day) (Thenganatt & Jankovic, 2013). Side effects usually are manifested by dyskinesia (abnormal involuntary movements) (Thenganatt & Jankovic, 2013). By contrast, some patients may benefit from dopamine antagonists (neuroleptics) such as clozapine or dopamine depleting drugs, a vesicular monoamine transporter 2 (VMAT2) inhibitor tetrabenazine, but their use can be restricted by side effects, such as weight gain or involuntary muscle movements (Bhidayasiri, 2006; Lubarr & Bressman, 2011) as well as tardive dyskinesia, akathisia, drowsiness, depression, insomnia, and parkinsonism (Jankovic, 2013; Thenganatt & Jankovic, 2013).

Anticholinergics

Anticholinergic agents are compounds that bind to the nicotinic and muscarinic acetylcholine receptors blocking the action of neurotransmitter acetylcholine in the central and peripheral nervous system. Most commonly used anticholinergics in the treatment of dystonia are muscarinic antagonists (antimuscarinics), with a first line choice trihexyphenidyl. Trihexyphenidyl has proven to be the most beneficial oral form of treatment for primary generalized and segmental dystonia (Burke, 1986; Jankovic, 2009a). Its efficacy was established in double-blind crossover prospective study three decades ago by Burke and colleagues (1986). Usually if trihexyphenidyl treatment starts at low dose (1 mg/day) and the dose is increased slowly, they are well tolerated. However, many patients may require high doses, up to 100 mg/day, and this may lead to dose-related side effects, e.g. drowsiness, hallucinations, impaired memory (Jankovic, 2006). Generally high doses are better tolerated by children than

adults. Although, anticholinergics are effective, they are non-selective and so non-specifically exert their effect on parasympathetic (cholinergic) nervous system and block the action of acetylcholine (ACh) on centrally and peripherally located muscarinic receptors, resulting in inhibition of involuntary movements of smooth muscles present mainly in the lungs, gastro-intestinal and urinary tract and at the same time, causing many side effects. Central side effects include confusion, memory loss, restlessness, insomnia, hallucinations, and drowsiness. Peripheral side effects include dry mouth, which is a severe problem preventing swallowing and speech, blurred vision, urinary retention, constipation and loss of appetite (Cloud & Jinnah, 2010; Lubarr and Bressman, 2011). While, these side effects can be reduced using locally active muscarinic agonists, such as pilocarpine eye drops for blurred vision, synthetic saliva, cevimeline or oral pilocarpine for dry mouth, and the peripheral cholinesterase pyridostigmine, for urinary retention (Bhidayasiri, 2006; Lubarr & Bressman, 2011), anticholinergics can still significantly affect quality of life and reduce compliance. For this reason, improved anticholinergic therapy is required that reduces the incidence of peripheral side effects while still reducing the severity of the dystonia.

1.1.2 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease (de Lau & Breteler, 2006). It was named after James Parkinson, who for the first time, based on observations of clinical features, described it as "Shaking Palsy" or "*Paralysis Agitans*" (Parkinson, 2002). PD is a common, chronic and progressive neurodegenerative disease (Lang & Lozano 1998; Massano & Bhatia, 2012). Clinical diagnosis is based on manifestation of cardinal symptoms consisting of resting tremor, rigidity, bradykinesia/akinesia (slowness or lack of movement) and postural and gait impairment (Jankovic, 2008; Massano & Bhatia, 2012). While conventionally PD was considered to be a motor complaint, nowadays it is considered to be more complex disorder accompanied by a diverse clinical features of non-motor symptoms, including sleep disturbances, cognitive decline (dementia), constipation, urinary dysfunction, fatigue and mood disorders (depression, anxiety) (Aarsland *et al.*, 1999; Fahn, 2010; Claassen & Kutscher, 2011; Massano & Bhatia, 2012). In recent years' attention and research have been focusing on trying to understand the influence

of these non-motor features in order to improve patients' quality of life (Chaudhuri *et al.*, 2011).

1.1.2.1 Pathology of PD

The motor symptoms in PD are the result of progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc). Neuropathologically PD is defined by the presence of intracytoplasmic inclusions termed Lewy bodies, found in remaining dopaminergic cells, thought to be a marker of dying neurons. Lewy bodies are made up mainly of neurofilament-like structure and contain ubiquitin and α-synuclein (Beal, 2001; Murakami *et al.*, 2004). In addition to nigral dopaminergic neuronal degeneration, there is a more widespread degeneration involving other neurotransmitter systems. Loss of noradrenaline neurons of the locus coeruleus, cholinergic neurons in the nucleus basalis of Meynert and serotonin neurons of the dorsal raphe nuclei has been detected along with, alternations in the function of glutamatergic and GABAergic pathways (Lang & Lozano 1998; Olanow, 2004; Hodaie *et al.*, 2007). Degeneration of the cells in these other brain regions has been suggested to underlie the occurrence of non-motor symptoms of the disease, such as depression due to the loss of noradrenergic neurons in the limbic system (Remy *et al.*, 2005).

Originally it was thought that the pathology of PD starts with the loss of dopaminergic cells in the substantia nigra, however, Braak and colleagues proposed a series of six disease progression stages based on α-synuclein and Lewy bodies presence, starting from the brain stem to the cerebral cortex (Braak *et al.*, 2003; Braak *et al.*, 2004). During the presymptomatic stages, (stage I and II) changes are confined to the dorsal motor nucleus and the olfactory bulb, related to loss of smell in patients prior to diagnosis, followed by the spread to the locus coeruleus and raphe nuclei. In the subsequent stages (stage III and IV), classic motor symptoms of PD start to appear and the pathology involves substantia nigra pars compacta, the midbrain and then the basal forebrain. The motor symptoms of PD usually appear when the pathology has reached stage where dopaminergic cell loss in SN is 50% and 80% of the striatal dopamine is depleted. In the final stages (stage V and VI), the pathological changes involve neocortical areas creating cognitive problems and dementia (Braak *et al.*, 2004). Despite this, the validity of Braak's hypothesis remains controversial, as often there is

no correlation between the symptoms and the progress of pathology of PD and the pathology does not always reflect the clinical severity of the disease (Burke et al., 2008; Brooks, 2010). Be that as it may, approximately 70% of post-mortem brains from PD sufferers follow the Braak staging (personal communication, Prof David Dexter Parkinson's UK Brain Bank, Imperial College London).

1.1.2.2 Epidemiology and aetiology

PD affects many people worldwide, regardless the ethnic group, with men more affected than women with a ratio 1.5:1 (Twelves *et al.*, 2003; Farrer, 2006). It is estimated that the prevalence of PD is about 0.3% in general population at the age 40 years and older, and 3% of people over the age of 65 years (Mayeux, 2003; Pringsheim *et al.*, 2014). These rates suggest that 7.5 million people worldwide are affected by PD. The mean age of onset of PD is around the 70, however, early-onset disease is seen in about 4% of patients before the age of 50 (Van Den Eeden *et al.*, 2003; Farrer, 2006).

The aetiology of PD still remains unknown. It is believed that both environmental and genetic factors have been considered as the possible underlying causes, with age and family history considered to be the major risk factors and contributors to development of PD (Jenner *et al.*, 2013). PD is mainly considered as an idiopathic disorder, however, there is also a genetic cause and a small number of sufferers (5–10%) have a familial PD (Thomas & Beal, 2007; Wirdefeldt *et al.*, 2011).

Many of the genes involved in aetiology of PD implicate molecular pathways involved in nigral degeneration, including protein aggregation, defective proteasomal degradation, oxidative stress and mitochondrial dysfunction suggesting common molecular mechanism which underlies PD (Litvan *et al.*, 2007).

Several genes are associated with inherited forms of the disease, such as PARK2 (parkin), PTEN-induced putative kinase 1 (PINK1), DJ-1, leucine-rich repeat kinase 2 (LRRK2) and the α-synuclein mutation (SNCA) (Wirdefeldt *et al.*, 2011) (Table 1.2). Many genes differ in terms of phenotype or pattern of inheritance, in case of familial PD, and those can be inherited as an autosomal dominant (SNCA or LRRK2) or autosomal recessive (PARK2 or PINK1) (Kruger *et al.*, 2002; Warner & Schapira, 2003; Lees *et al.*, 2009). Nonetheless, there is also evidence showing that some genes,

such as LRRK2 mutation, can be involved in both familial and idiopathic PD (Paisán-Ruíz *et al.*, 2004; Gilks *et al.*, 2005).

α-synuclein was the first gene found that the mutations were associated with familial early onset PD and verified as a major component of Lewy bodies. Mutations of α-synuclein lead to its structural modifications causing misfoldings and aggregations and this plays a role in pathogenesis of PD (Kruger *et al.*, 2002).

PARK2 is autosomal recessive disease and is associated with juvenile PD, however, in heterozygotes is associated with adult-onset PD (Foroud *et al.*, 2003).

Mutation of PINK1 gene can be responsible for a rare familial form of PD. Although, its actual role is not clarified, it seems to encode a mitochondrial protein and could have a role in neuroprotection (Valente *et al.*, 2004) (Table 1.3)

One of the most common mutation LRRK2 gene is G2019S and it affects majority of Portugese, Ashkenazi Jews, North African Arabs PD patients (Fahn, 2010).

Furthermore, mutations in the gene encoding for glucocerebrosidase (GBA) is a significant risk factor for PD. GBA is a lysosomal enzyme catalysing the hydrolysis of the membrane glycolipid, glucocerebroside, to glucose and ceramide (Velayati *et al.*, 2010; Beavan & Schapira, 2013). Deficiency in the glucocerebrosidase activity are associated with the autosomal recessive lysosomal storage disorder, Gaucher disease (GD), which frequently affects Ashkenazi Jewish population (Beavan & Schapira, 2013). Mutations of GBA have been implicated in PD through lysosomal deficiency and autophagic dysfunction. This causes reduction of α-synuclein degradation leading to the accumulation of undesirable misfolded proteins and as a consequence further disruption of the autophagic system (Velayati *et al.*, 2010). The phenotype is indistinguishable clinically, pathologically and pharmacologically from sporadic PD, except the mutation in GBA (Beavan & Schapira, 2013). While heterozygous mutations of GBA lead to GD, both homozygous and heterozygous mutations increase the risk of developing PD by 20 to 30-fold. Approximately 5 – 10% of PD sufferers have a GBA mutation (Velayati *et al.*, 2010; Beavan & Schapira, 2013).

In addition to the genetic factors, environmental agents are thought to contribute to aetiology of the disease. Numerous epidemiological studies have indicated that use and exposure to pesticides (paraquat), and herbicides (rotenone) have made farmers more susceptible to develop PD (Hubble *et al.*, 1993; Priyadarshi *et al.*, 2001; Monte, 2003). Also, drinking well water, living in rural areas, exposure to heavy metals,

including copper, iron, manganese or lead, and some occupations, such as mining and welding, contribute to aetiology of the disease (Priyadarshi *et al.*, 2001; de Lau & Breteler, 2006).

Table 1.3 Genes underlying monogenic parkinsonism.
AD – autosomal dominant; AR – autosomal recessive.
Adapted and modified from Klein & Westenberger (2012).

PARK locus	Gene	Gene locus	Mode of inheritance
PARK1	SNCA	4q21-22	AD
PARK2	Parkin	6q25.2-q27	AR
PARK3	Unknown	2p13	AD
PARK4	SNCA	4q21-q23	AD
PARK5	UCHL1	4p14	AD
PARK6	PINK 1	1p35-36	AR
PARK7	DJ-1	1p36	AR
PARK8	LRRK2	12q12	AD
PARK9	ATP13A2	1p36	AR
PARK10	Unknown	1p32	Not clear
PARK11	GIGYF2	2q37	AD
PARK12	Unknown	Xq21-q25	Not clear
PARK13	HTRA2	2p12	Not clear
PARK14	PLA2G6	22q13.1	AR
PARK15	FBXO7	22q12-q13	AR
PARK16	Unknown	1q32	Not clear
PARK17	VPS35	16q11.2	AD
PARK18	EIF5G1	3q27.1	AD

1.1.2.3 PD Therapy

Regardless of all the advances and effort made in trying to understand the pathophysiology of the disease, PD remains incurable and there are no proven treatments to slow the disease progression. Surgical techniques, such as pallidotomy and deep brain stimulation (DBS) of internal segment of the globus pallidus (GPi) and subthalamic nucleus (STN) have broadened the therapeutic options and proved to be effective in providing improvement in cardinal symptoms of PD, as well as reducing motor complications associated with dopaminergic treatment (Olanow, 2004; Volkmann, 2004). Despite that, currently the treatment is largely symptomatic and relies mainly on pharmacotherapy targeting the motor symptoms. The treatment is mainly focused on replacement of dopamine, however, there are other useful forms of pharmacotherapy.

L-DOPA

Since the first description of the beneficial effect of high dosage of L-DOPA in 1968, it remains the most effective and a gold standard symptomatic treatment for PD (Barbeau, 1969; Fahn, 2008). Orally administered L-DOPA is metabolised into dopamine in the periphery, and only 1% of the drug crosses the blood brain barrier, reducing the efficacy and resulting in side effects, such as nausea or vomiting, therefore L-DOPA is given in conjunction with dopa decarboxylase inhibitors (DDCI) – benserazide or carbidopa (Schapira, 2005; Hauser, 2009). The combination of treatment results in increased plasma half-life of L-DOPA, which in turn increases efficacy in the brain and reduces peripheral side effects of dopamine (nausea, vomiting) (Hauser, 2009).

In addition to that, other treatments are given as an adjunct to L-DOPA to increase its effects and minimize the side effects. Catechol-O-methyltransferase (COMT) inhibitors (entacapone, tolcapone) decrease metabolism of L-DOPA to 3-O-methyldopa, only the latter having an effect centrally. The result is a small but effective prolongation of the duration of the central effects of L-DOPA (Smith *et al.*, 2012).

Monoamine oxidase (MAO)-B inhibitors (selegiline, rasagiline) increase the effect of dopamine in the brain by preventing the enzymatic breakdown of endogenous dopamine into 3,4-dihydroxyphenylacetic acid (DOPAC) thus enhancing levels of

both endogenous and L-DOPA-derived dopamine (Fahn, 2008; Goldenberg, 2008; Smith *et al.*, 2012).

Amantadine, originally an antiviral drug, has mild antiparkinsonian properties. It inhibits excessive stimulation of glutamate receptors in the brain by acting as a weak NMDA receptor antagonist, increases dopamine release and blocks dopamine uptake into the nerve terminals and has anticholinergic properties (Hallett *et al.*, 2006).

Amantadine therapy results in number of unwanted effects, including nausea and insomnia, increased risk of seizure, and not all patients respond to the treatment. However, amantadine has been shown to reduce the severity of dyskinesia and now is the only clinically prescribed drugs to reduce these unwanted movements and so is mainly used in combination with L-DOPA in affected patients if they tolerate the high doses (Thomas *et al.*, 2004).

While short term use of L-DOPA provides exceptional symptomatic relief of the motor symptoms, chronic use is associated with the development of motor complications, such as "wearing off" (a decrease in duration of L-DOPA effect), "on-off" (changeable switching between beneficial response and immobility in PD state) and dyskinesia (abnormal involuntary movements) during "on" periods (Iravani & Jenner, 2011). In the early stage of the disease, L-DOPA can be stored in synaptic vesicles in presynaptic dopamine terminals in the striatum, where is converted to dopamine and then slowly released. However, the prolonged use of L-DOPA, pulsatility in plasma and brain exposure due to its short half-life, as well as the progression of the disease, result in loss of dopaminergic neurons in the striatum are all though to contribute to the shortening duration action of L-DOPA. Thus, it becomes harder to predict the duration of effect of a single dose resulting in "wearing off" and extended "off" phases causing immobility of a patient. The reoccurring symptoms require more frequent drug administration, which in turn requires intermittent L-DOPA administration resulting in more pulsatile stimulation and disturbances of postsynaptic dopamine receptor regulation in the striatum, causing dyskinesia. The rate in which dyskinesia develops depends on the striatal denervation and pulsatile nature of the oral L-DOPA treatment (Schapira, 2005; Iravani & Jenner, 2011).

Dopamine agonists

A variety of dopamine agonists are used in the treatment, either as a monotherapy or as an adjunct to other treatments, and are classified as ergot (bromocriptine, lisuride, pergolide and cabergolide) and non-ergot derivatives (apomorphine, pramipexole, ropinirole and rotigotine) (Jenner, 2003a; Schapira, 2005). They directly stimulate postsynaptic dopamine receptors in the striatum (Jenner, 2003). Dopamine agonists bind to different classes of dopamine receptors, however, most commonly used agents activate mainly D2 and D3 receptors. Despite that, pergolide and apomorphine show affinity for D1 receptor, a target of anti-parkinsonian treatment (Jenner, 2003). With the exception of apomorphine, their half-life tends to be longer than L-DOPA, therefore they usually have longer effect depending on type of the compound. Similarly as with L-DOPA, dopamine agonists produce number of side effects, including hypotension, nausea and vomiting (Stowe *et al.*, 2008), as well as psychiatric disturbances (hallucinations and confusion), and impulse control disorders (hypersexuality, pathological gambling, compulsive shopping or binge eating), requiring cessation of therapy (Lees *et al.*, 2009).

Anticholinergics

Anticholinergics were the first drugs to be used in the treatment of PD. The antiparkinsonian effect of anticholinergics was described in 1867 by Ordenstein who defined effect of belladonna alkaloids, naturally occurring anticholinergics, in the treatment of parkinsonian tremor (Goetz, 2011). Decades later, in the 1940s, first synthetic anticholinergic trihexyphenidyl was introduced.

The mechanism of anticholinergics action is not well understood, however, their use is intended to improve the striatal imbalance between the dopaminergic and cholinergic system by blocking the action of acetylcholine (ACh) and to reduce motor symptoms (Duvoisin, 1967; Schapira *et al.*, 2006). Cholinergic and dopaminergic system are closely related, and ideally, they should be in persistent balance, however, in PD reduced production of dopamine caused by degeneration of nigral neurons leads to the imbalance between both neurotransmitters (Duvoisin, 1967).

Centrally acting anticholinergics, such as trihexyphenidyl and benztropine, have been used for many years, however, their use have declined in recent years mainly after the introduction of L-DOPA and dopamine agonist, as the latter have proven to be more

useful in alleviating the PD symptoms. Nevertheless, anticholinergics are still used, as they show useful effect in reducing some of the troublesome motor symptoms, particularly tremor (Fox, 2013). They are often used in younger PD patients (≤60 years old) as a monotherapy, before the L-DOPA treatment commences. They are also used in combination with other drugs where L-DOPA treatment can possibly be delayed, consequently extending the use of the "gold standard drug", and also are used together with L-DOPA. Anticholinergics are also useful in the treatment of dystonia in PD, which is seen in early stages of young-onset of PD, and commonly seen as an early morning "wearing off" occurrence (Brocks, 1999; Katzenschlager *et al.*, 2003). In recent years, a review study looking at the use and effect of anticholinergics in the treatment of motor symptoms of PD was conducted by Cochrane (Katzenschlager *et al.*, 2003). In this study, the results from nine clinical trials with the use of various anticholinergics were compared. Tremor was the main outcome measure, and in five studies there was a significant improvement of tremor in patients administered anticholinergic compared to placebo. Additionally, bradykinesia and rigidity were found to be significantly improved in patients receiving anticholinergic therapy (Katzenschlager *et al.*, 2003).

Regardless of the positive effect, currently used anticholinergics are non-selective, hence their clinical potential is limited due to the large number of unfavourable central and peripheral side effects, which are particularly poorly tolerated in elderly patients, as they are more susceptible to memory impairments, sedation, confusion, hallucinations, urinary retention, dry mouth or blurred vision (Schapira *et al.*, 2006; Langmead *et al.*, 2008; Pirtosek, 2009; Fox, 2013). Therefore, the side effects are the main fundamental reason of their reduced use in patients. Since there is an evidence that anticholinergics can improve motor symptoms of PD (Katzenschlager *et al.*, 2003) the research should focus on finding more selective compounds, which would bring benefit of reducing cholinergic activity and controlling motor disability with the benefit of fewer side effects and greater tolerability.

1.2 Cholinergic function in the basal ganglia

Clearly anticholinergics play an important role in the treatment of both dystonia and PD, although in both diseases their use is limited due to unpleasant peripheral side effect that affect compliance. There is, therefore, an unmet need for improved anticholinergic therapy. In order to address this, it is important to have an understanding of the role of ACh in the control of movement, in particular in basal ganglia function.

1.2.1 The neurochemistry of the basal ganglia

The basal ganglia (BG) are a group of subcortical nuclei which, together with the cortex and brain stem, coordinate and incorporate a range of actions, including motor, cognitive and motivational processes (Grillner *et al.*, 2013). The BG are comprised of input and output nuclei (DeLong & Wichmann, 2007; Lanciego *et al.*, 2012). The input nuclei encompass the structures that obtain information from other areas of the brain, and consist of striatum, subdivided for caudate nucleus and putamen, and subthalamic nucleus (STN). The input pathways comprise of the nigrostriatal dopaminergic pathway, the corticostriatal glutamatergic pathway, the thalamostriatal glutamatergic pathway, the cholinergic and glutamatergic tracts from the pedunculopontine nucleus, the serotoninergic inputs from the raphe nuclei, and a sparse noradrenergic innervation from the locus coeruleus. The output nuclei send information to the thalamus and consists of internal and external segment of the globus pallidus (GPi and GPe, respectively), substantia nigra pars reticulata (SNr) and substantia nigra pars compacta (SNc) (Fig. 1.3.) (Lanciego *et al.*, 2012). These structures are functionally connected with cerebral cortex and thalamus and involved in the cortico–basal ganglia–thalamocortical loop (Alexander *et al.*, 1986; Parent & Hazrati, 1995a). The main function of the BG is the execution of movement by balanced activity of the direct and indirect striatal output pathways (Gerfen, 2000). Disruptions of the basal ganglia circuits is related to the large range of psychiatric and neurological diseases, including dystonia and PD (Obeso *et al.*, 2008).

The main integration center of the BG is the striatum, which receives the afferents from various cortical and subcortical structures and project to numerous BG nuclei, in particular the internal and external segments of the globus pallidus (Crittenden &

Graybiel, 2011). Excitatory glutamatergic connections from cerebral cortex and thalamus enter the striatum via corticostriatal and thalamostriatal pathway respectively, and terminate at the projection neurons called medium spiny neurons (MSN). MSN constitute nearly 95% of all striatal neurons and are inhibitory using GABA as the neurotransmitter (Rouse *et al.*, 2000; Lanciego *et al.*, 2012). They form the basis of the GABAergic neostriatal output, innervating the structures of BG and making up the start of the direct and indirect output pathways (DeLong & Wichmann, 2009). In addition, the activity of the striatum is also controlled by dense projections from midbrain dopaminergic neurons (SNc and ventral tegmental area) as well as giant aspiny cholinergic and GABAergic interneurons (Kawaguchi *et al.*, 1995; Benarroch, 2012).

1.2.1.1 The basal ganglia in the control of movement

As mentioned earlier, components of the BG form a closed circuit which leads to differential effects. Numerous neurotransmitters can be distinguished within the circuits and their release results in transmission of signals. These are GABA, acting as inhibitory neurotransmitter, excitatory glutamate (GLU) and dopamine (DA), which has both excitatory and inhibitory function. In addition, ACh has a major role in controlling the direct and indirect pathways by acting directly on MSN, presynaptic effects on corticostriatal pathways and interaction with dopamine (DeLong & Wichmann, 2009).

The direct efferent pathway from the striatum is made up of GABAergic MSN projection directly to the GPi/SNr which also contain the neuropeptide dynorphin and substance P as co-transmitters. This pathway is controlled by glutamatergic inputs from the cortex and dopaminergic inputs from the SNc, the latter stimulated by excitatory dopamine D1 receptors. Stimulation of the direct pathway, either by glutamate or dopamine, results in the promotion of movement (Fig. 1.2 A) (DeLong, 1990; Parent & Hazrati, 1995a; DeLong & Wichmann, 2007).

The indirect pathway is made up of striato-pallidal efferent MSN which project to the GPe which are GABAergic and contain encephalin as co-transmitter. These then connect with GABAergic projections to the STN, synapsing with glutamatergic outputs to the GPi/SNr. The striatal efferents are controlled by excitatory

glutamatergic inputs from the cortex, and dopaminergic inputs from the SNc, the latter connecting to inhibitory D2 receptors (Hauber *et al.*, 1998). These inputs have opposing effects such that stimulation of the indirect pathway by glutamate results in inhibition of movement, whereas dopamine, via inhibitory D2 receptors, results in the promotion of movement (Fig. 1.2 A) (DeLong, 1990; Parent & Hazrati, 1995a; Parent & Hazrati, 1995b).

1.2.1.2 Interneurons

Several classes of local-circuit neurons, termed interneurons, can be found in the striatum, and these are cholinergic, GABAergic and tyrosine-hydroxylase-immunoreactive (Kawaguchi *et al.*, 1995; Zhou *et al.*, 2002; Kreitzer, 2009).

The GABAergic interneurons are divided into three subtypes. Firstly, parvalbumin-immunoreactive interneurons which express the calcium-binding protein parvalbumin. They are characterized by a fast-spiking phenotype and short-lasting action potential. Secondly, the calretinin-immunoreactive interneurons which express calcium-binding protein calretinin. There are three or four different types of these interneurons, however, their exact function is not known. Thirdly, a set of interneurons that are immunoreactive for somatostatin and nitric oxide, known as persistent and low threshold spike (PLTS) interneurons that are able to produce calcium-dependent low threshold spikes and large and stable sodium potentials as a response to synaptic stimulation (Kawaguchi *et al.*, 1995; Beatty *et al.*, 2012; Lanciego *et al.*, 2012). Studies on rodents suggested that there is an interaction between these, the cholinergic interneurons (ChI) and parvalbumin-positive fast spiking interneurons (Chang & Kita, 1992; Lanciego *et al.*, 2012) resulting in suppression of striatal GABA release most likely through muscarinic M2 receptors (Marchi *et al.*, 1990; Raiteri *et al.*, 1990; Bernard *et al.*, 1992).

Four types of striatal tyrosine-hydroxylase-immunoreactive interneurons have been identified using electrophysiological studies, however, their functional role is unknown (Ibáñez-Sandoval *et al.*, 2010).

From all of the interneurons, the large aspiny cholinergic interneurons (ChI) are the most abundant (1–3% of total striatal neurons) (Aosaki *et al.*, 1995; Kawaguchi *et al.*, 1995; Zhou *et al.*, 2002). They have large cell bodies (20 – 50 μm diameter) and extensive axonal fields (Kawaguchi *et al.*, 1995). Their electrophysiological properties

include long lasting action potential and continuous and persistent spontaneous firing activity, hence they are termed as tonically active neurons (TAN) (Aosaki *et al.*, 1995; Kawaguchi *et al.*, 1995; Zhou *et al.*, 2002). They have burst-pause pattern during their firing, and the burst is mediated by excitatory glutamatergic inputs, whereas the pause is triggered by dopaminergic connections from the SNc, mediated by inhibitory D2 receptors (Aosaki *et al.*, 2010). Whereas their axonal aborizations are mainly restricted to the matrix of the striatum, the ChI possess large dendrites which are spread across both the matrix and striosomes of the striatum, and they integrate with the MSN of both the direct and indirect pathways (Kawaguchi *et al.*, 1993). These features suggest that ACh is tonically released from the interneuron, and this is controlled by cholinergic receptors in the striatum. ChI express both dopamine D2 and D5 receptors and muscarinic M2 and M4 receptors (Hersch *et al.*, 1994). Activation of D2 receptors mediates inhibition of Na^+ channels that reduces excitability, whereas activation of D5 results in depolarization of ChI through cAMP-dependent mechanism (Maurice *et al.*, 2004). Muscarinic M2/M4 autoreceptors mediate a negative feedback control on ACh release by reducing the opening of Cav2 Ca^{2+} channels connected to exocytosis and by promoting opening of Kir3 K^+ channels that cause hyperpolarization and decrease of Ca^{2+} channel opening (Calabresi *et al.*, 1998; Ding *et al.*, 2006; Pisani *et al.*, 2007). ChI are the synaptic targets of striatal afferents from the cerebral cortex, thalamic nuclei, substantia nigra, locus coeruleus, dorsal raphe nuclei and MSN (Aosaki *et al.*, 2010; Bonsi *et al.*, 2011). They receive glutamatergic input, producing excitatory postsynaptic potentials, from the thalamic nucleus and in low amount from the cerebral cortex.

In turn, excitability of the ChI can be elevated by D1/D5 receptor activation (Aosaki *et al.*, 2010). The connection between the ChI and the MSN of the direct and indirect pathway result in an important modulatory role of movement, mainly via M1 and M4 muscarinic receptors (Breakefield *et al.*, 2008).

1.2.1.3 Basal ganglia in dystonia

The development of hyperkinetic movement disorders such as dystonia occurs due to the changes in neuronal activities in the BG and thalamus, changes in the metabolic and excitability in the cerebral cortex and loss of inhibition to spinal and brain stem reflexes leading to the changes in muscle activity during movement (Berardelli *et al.*,

1998; Quartarone & Hallett, 2013). Thus, the increase in the inhibitory output from the striatum to GPe and GPi via indirect and direct pathways, respectively (Vitek, 2002). The overactivity of the spiny projection neurons in the direct pathway leads to disinhibition of motor circuits to facilitate movements (Fig. 1.2 C) (Gittis & Kreitzer, 2012). Additionally, elevated cortical excitability and changes in spinal and brainstem reflexes have been documented (Vitek, 2002).

1.2.1.4 Basal ganglia in PD

Basal ganglia function is altered in PD due to the loss of dopaminergic afferents which control the direct and indirect striatal output pathways. The loss of excitatory input to the direct pathways results in understimulation, and ultimately reduced thalamo-cortical stimulation. The reduced inhibitory effect of dopamine on the indirect pathway increases the activity of the striato-GPi pathway which, via connection to the STN, GPe and thalamus, also reduces thalamo-cortical activity. Thus, the result of the loss of dopamine in PD causes reduced activity of the motor cortex, and hypokinesia (DeLong & Wichmann, 2010; Lanciego *et al.*, 2012). As described above, treatment of PD with L-DOPA and dopamine agonists can reverse these motor deficits by replacing the activity of the DA receptors (Fig. 1.2 B) (Schapira, 2005; Hauser, 2009).

1.2.1.4.1 Basal ganglia with long-term L-DOPA (in dyskinesia)

In dyskinesia, treatment with L-DOPA causes excessive stimulation of D1 and D2 receptors, leading to underactivity of the indirect output pathway and overactivity of the direct output pathway. This decreases the GABAergic input to the thalamus and increase firing of the thalamic neurons, resulting in an involuntary movement (Fig. 1.2 C) (DeLong, 1990; DeLong & Wichmann, 2007).

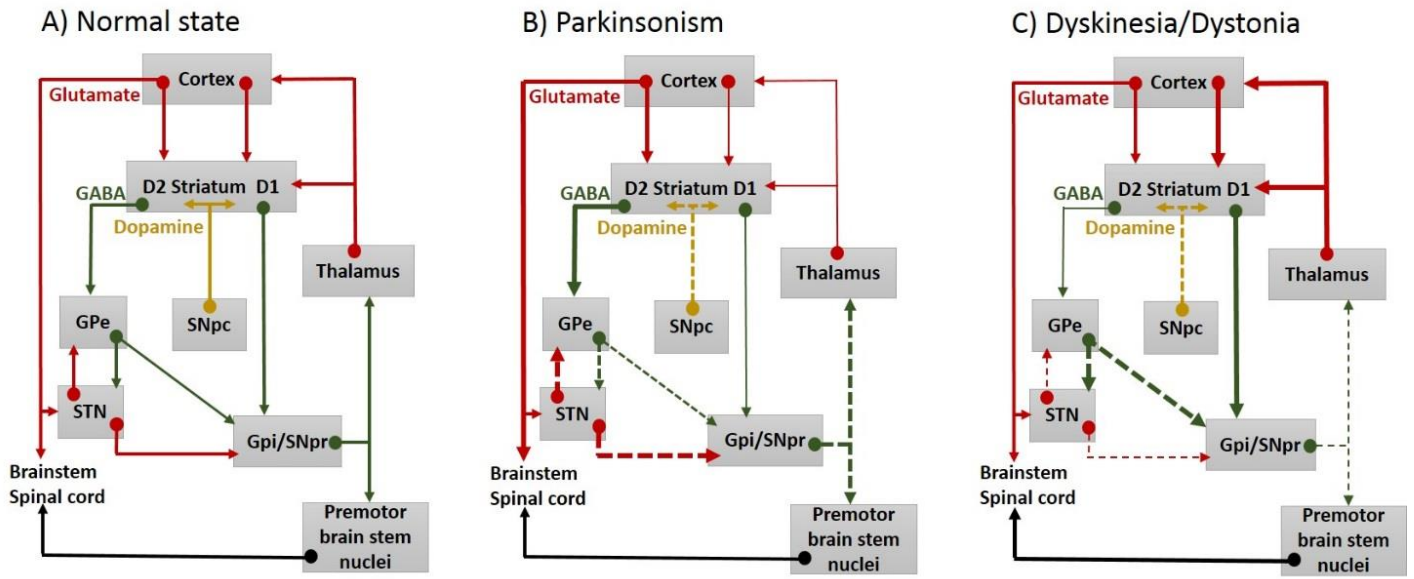


Figure 1.2 The basal-ganglia-thalamocortical circuits.
Normal conditions (a), parkinsonian (b) and in L-DOPA-induced dyskinesia (c). In (b) and (c) changes in the average activity rate of specific projection pathways are shown as thickening (increased activity) or thinning (decreased activity) compared to the normal state. Alterations in firing pattern are represented by dashed lines. The striatum and STN provide the input layer for incoming cortical information to the basal ganglia. The GPi and SNr provide the output layer communicating with the rest of the brain. These structures exert strong inhibitory control on their projection targets in the thalamus and the brainstem and this tonic inhibitory output must be released to enable normal movements to occur. The striatum exerts the opposite influences via two classes of efferent neurons, the D1 receptor of direct pathway, positively modulated by dopamine, and D2 receptor of indirect pathway, negatively modulated by dopamine. Additionally, the STN receives excitatory direct input from the cerebral cortex through hyperdirect pathway. The loss of dopamine in PD (b) causes an imbalance of the activity of the two pathways and their corresponding cortical inputs. During the expression of dyskinesia and dystonia (c) the direct pathway is overactive resulting in reduction of output of the GPi and the thalamic input to the cortex is enhanced (Berardelli *et al.*, 1998; Cenci, 2007). (GPe, external segment of globus pallidus, GPi, internal segment of globus pallidus, SNc, substantia nigra pars compacta, SNr, substantia nigra pars reticulata, STN, subthalamic nucleus. Adapted and modified from Cenci (2007).

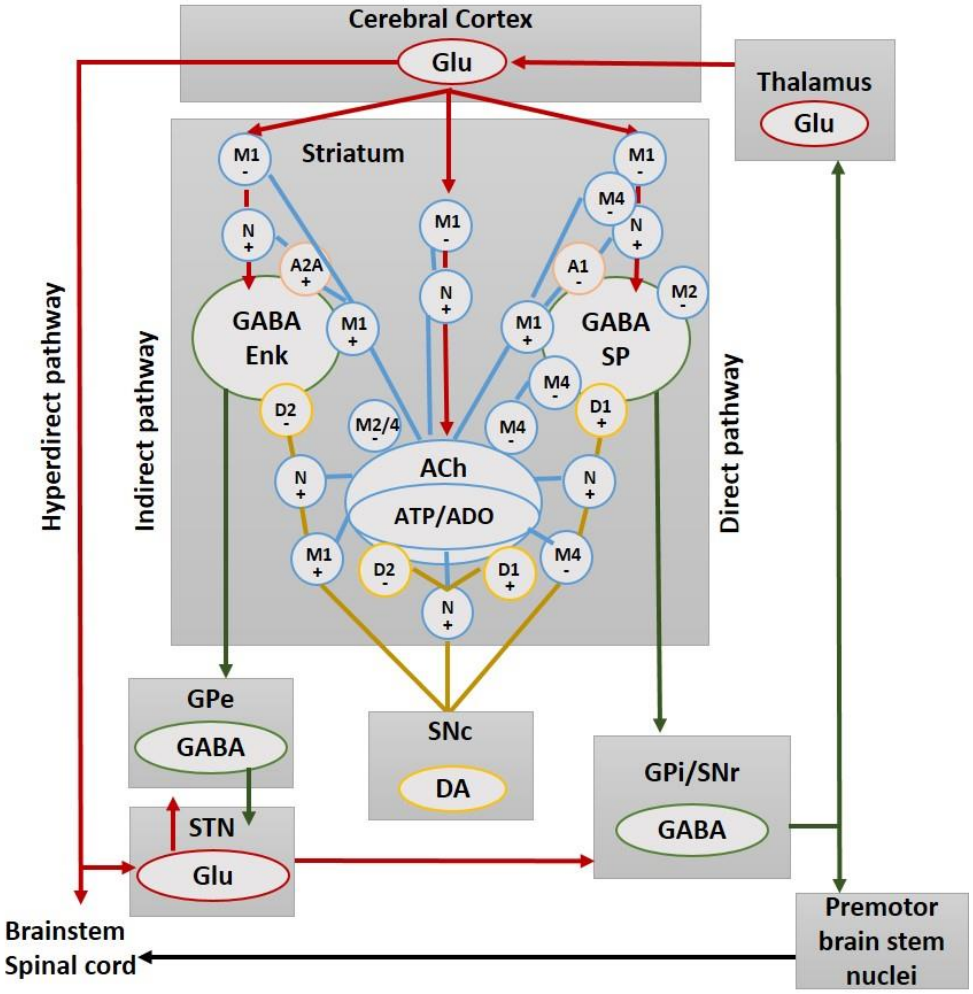


Figure 1.3 The simplified basal-ganglia-thalamocortical circuits under normal conditions.
Figure representing the innervation of the striatum by the nigrostriatal dopamine system and mediation of excitatory and inhibitory influence, through dopamine D1 and D2 receptors and ACh muscarinic receptors (M1, M2, M4), of direct and indirect GABAergic striatal output pathways to the globus pallidus internus (GPi) and substantia nigra pars reticulata (SNr), respectively. ACh modulate DA and Glu release by acting on presynaptic nicotinic receptors located on striatal dopaminergic terminals, as well as via M1 receptors located on neurons in the substantia nigra pars compacta (SNc). ACh also acts on M1 and M2/M4 receptors on striatal GABAergic spiny neurons, reduces Glu release from glutamatergic terminals acting on M2 receptors, and controls its own release by acting on M2/M4 autoreceptors. Nigrostriatal DA may also interact with striatal cholinergic interneurons to mediate corelease of adenosine triphosphate (ATP) and adenosine (ADO) to act on A1 and A2a receptors. ACh, acetylcholine; GABA, gamma-aminobutyric acid; Glu, glutamate; DA, dopamine; Enk, enkaphalin; SP, Substance P; + and – represents the excitatory and inhibitory influence of each receptor subtype. Adapted from Calabresi *et al.*, (2006); Lester *et al.*, (2010).

1.3 The role of acetylcholine in the basal ganglia

Balance between the two main neurotransmitters acetylcholine and dopamine is vital in maintaining normal function of basal ganglia. In the striatum, cholinergic neurotransmission is regulated by release of acetylcholine from ChI (Ponterio *et al.*, 2012). Acetylcholine exerts its effect though nicotinic and muscarinic receptors which both are found in abundance in basal ganglia and contribute to basal ganglia function (Wess *et al.*, 2007).

1.3.1 Nicotinic receptors

Nicotinic receptors are ligand-gated ionotropic cholinergic receptors composed of five subunits arranged symmetrically forming a central pore (Hurst *et al.*, 2013). They are activated by endogenous ACh, exogenous nicotine or other ligand. There are two subfamilies of nicotinic subunits: alpha (α2 – α10) and beta (β2 – β4) (Gotti *et al.*, 2006). They can be accumulated and form homomeric or heteromeric subunits combinations which define characteristic pharmacological and biophysical properties of the receptor (Gotti *et al.*, 2006). Alpha subunits are necessary for binding of acetylcholine, and beta subunits can alter binding and dissociation constant of nicotinic agonists and antagonists, thus regulating their activity (Wang *et al.*, 1996).

The most highly expressed nicotinic receptors in the striatum are α4, α6, α7, β2 and β3, however, other subtypes can also be present but in lower levels (Zoli *et al.*, 2002). α4β2* receptors are localized on dopaminergic neurons, serotonergic efferents, striatal GABAergic and ChI. Additionally, other subtypes of nicotinic receptors are expressed on dopaminergic terminals, including α6β2β3, α6α4β2β3 and α4α5β2 (Zoli *et al.*, 2002; Champtiaux *et al.*, 2003). Striatal non-dopaminergic terminals express α2α4β2, whereas corticostraital afferents, glutamate terminals, and GABA neurons can express α7* subunits on nerve endings (Marchi *et al.*, 2002; Zoli *et al.*, 2002). They have a role in synaptic plasticity, mainly LTP, due to their Ca^{2+} permeability and initiation of Ca^{2+}dependent processes (Dajas-Bailador & Wonnacott, 2004). Nicotinic receptors control release of dopamine on nigrostriatal terminals (Threlfell *et al.*, 2012) and have been the target for new therapies for PD, as agonists have been shown to reduce dyskinesia in animal models (Quik *et al.*, 2015).

1.3.2 Muscarinic receptors

Muscarinic receptors belong to the G-protein coupled receptors (GPCRs) (Caulfield, 1993; Jinnah & Hess, 2008). Five types of muscarinic receptors (M1 – M5) have been cloned, characterised and divided into two distinct groups according to the signal transduction. M1, M3 and M5 receptors couple via G_q/G_{11} proteins to activate phospholipase C (PLC) and mobilise intracellular calcium, whereas M2 and M4 receptors inhibit activation of adenylyl cyclase (AC), by coupling to G_i/G_o, which results in reduction of the intracellular concentration of cyclic AMP (cAMP) (Langmead *et al.*, 2008).

1.3.2.1 Location and major functions of muscarinic receptors

Muscarinic receptors are broadly distributed in various regions of the central and peripheral nervous system where they are responsible for regulation of vital physiological processes (Wess *et al.*, 2007; Nathanson, 2008). For instance, in the CNS they are responsible for cognition, memory, behaviour, motor control and sensory function, and in the periphery, they regulate the heart rate, smooth muscle contraction and stimulation of glandular secretion (Caulfield, 1993; Wess, 2004; Wess *et al.*, 2007). Moreover, different tissues may express more than one subtype of the receptor, which makes it difficult to determine the exact location and function of the particular receptor (Wess *et al.*, 2007) (Table 1.4).

The M1, M4 and M5 receptors are predominantly located in the CNS, whereas the M2 and M3 receptors are mainly located in the CNS and in peripheral tissues (Wess *et al.*, 2007). In the CNS, depending on subtype and neuron where they are present, the muscarinic receptors are located pre- and postsynaptically (Nathanson, 2008).

M1 receptors are reported to be largely found in the CNS, in the cerebral cortex, striatum, hippocampus and thalamus where are found postsynaptically and are involved in learning and memory processes (Caulfield, 1993). Muscarinic agonists or acetylcholinesterase inhibitors enhance cholinergic receptor activation which results in improvement of cognitive deterioration, thus selective M1 agonism has been proposed as a therapeutic approach in dementia, including Alzheimer's disease, and other memory and cognitive impairments associated with age (Fisher *et al.*, 2003; Terry & Buccafusco, 2003). Low levels of M1 subtype of muscarinic receptors have

been also associated with schizophrenia, with links to changes in long-term synaptic plasticity, auditory and visual hallucinations and memory deficits (Dean *et al.*, 2016). In the periphery, they are mainly present in secretory glands (salivary, gastric, lacrimal), where they are responsible for increase of saliva and gastric acid secretion (Caulfield, 1993).

Muscarinic M3 receptors centrally are identified in the cerebral cortex and hippocampus and a very low amount of M3 is expressed in the striatum (Hersch *et al.*, 1994; Eglen *et al.*, 1996). Peripherally they are found in the exocrine glands (gastric, salivary), smooth muscle (gastrointestinal, eye, airways and bladder) and blood vessels (endothelium) (Eglen *et al.*, 1996). Generally, their role is to mediate contraction of smooth muscles, including respiratory and gastrointestinal, where they help to promote gastric emptying and contraction of the gut, contraction of the iris and stimulate secretion from the glands (Eglen *et al.*, 1996; Stengel *et al.*, 2002; Tobin *et al.*, 2002). M5 receptors are distributed in the cortex, hippocampus, substantia nigra, ventral tegmental area (VTA), region involved in reward and addiction and low amount in the striatum (Hersch *et al.*, 1994; Eglen, 2006; Langmead *et al.*, 2008). Blockade of M5 receptors may be effective in the treatment of drug dependence by inhibition of dopamine release in the VTA (Langmead *et al.*, 2008). Peripherally they are expressed in salivary gland and in the eye (Langmead *et al.*, 2008).

Muscarinic M2 receptors in the CNS are expressed in the brainstem, thalamus, cerebral cortex, hippocampus and striatum (Eglen, 2006; Langmead *et al.*, 2008). In the cortex, they can be found as cholinergic autoreceptors and in the striatum they are located on cholinergic interneurons (Bernard *et al.*, 1998; Mesulam, 1998; Piggott *et al.*, 2003). Function of autoreceptors can be reduced by selective blockade of M2 resulting in an increases cholinergic overflow. Additionally, selective antagonism of M2 receptors may provide a therapeutic approach in treatment of schizophrenia, as in the caudate putamen M2 receptors acts as inhibitory heteroreceptors on dopaminergic terminals (Eglen, 2006). Nevertheless, the major role of M2 receptors is in periphery, where they are abundantly expressed in the heart muscle, particularly postsynaptically in the myocardium, where they decrease heart rate and force of contraction (Langmead et al., 2008). M2 receptors are also co-expressed with M3 receptors in smooth muscles, including gastrointestinal (Eglen *et al.*, 1996) and in the bladder detrusor muscle (Abrams *et al.*, 2006).

By contrast, muscarinic M4 receptors are mainly located centrally and are abundantly expressed in the striatum and at lower levels pre- and postsynaptically in the cortex and hippocampus (Rouse *et al.*, 1999; Piggott *et al.*, 2003). Furthermore, they are involved in motor control and may play a role in cognition. It has been suggested that muscarinic M4 receptors may play a role in restoring the balance between the DA and ACh in PD and dystonia. Preclinical studies with the use of various animal models showed that blockade of M4 receptors increases locomotor activity, however, the blockers used in these studies were not highly selective for M4 subtype (Gomeza *et al.*, 1999a; Mayorga *et al.*, 1999; Karasawa *et al.*, 2003; Betz *et al.*, 2007).

Table 1.4 Central and peripheral location and main functions of muscarinic M1 – M5 receptors.
Adapted from Eglen (2006); Langmead *et al.* (2008).

Muscarinic receptor	Location	Main function
M1	Cortex, hippocampus, striatum, thalamus, glands (gastric, lacrimal, salivary)	Involved in learning and memory processes; increase secretion of gastric acid, saliva, tears
M2	Brainstem, thalamus, cortex, hippocampus, striatum, heart, smooth muscle	Regulate heart rat
M3	Cortex, hippocampus, exocrine glands (gastric, salivary), smooth muscle (GI tract, eye, airways, bladder), blood vessels (endothelium)	Mediate contraction of smooth muscles (respiratory, GI), contraction of iris, stimulate gland secretion
M4	Striatum, cortex, hippocampus	Involved in locomotor activity and cognition
M5	Cortex, hippocampus, substantia nigra, ventral tegmental area, salivary glands, eye	Involved in cognition, role in saliva secretion

As described previously cholinergic antagonist are widely used clinically in the treatment of many disorders. Due to the action of anticholinergics on peripherally located muscarinic receptors, patients experience unpleasant side effects, including dry

mouth (mediated mainly by M3 and with less extend by M1 receptor), decreased sweating (M3 receptor), constipation (M3 receptor), urinary retention (M3 receptor), dilated pupils (M3 receptor), blurred vision (M1 and M4 receptors) and increased heart rate (M2 receptor). Although, these symptoms may not appear serious, eventually they may lead to more serious medical complications, such as gums ulceration, respiratory complications or even myocardial infarction (Lieberman, 2004). With this regard, although anticholinergics are a useful treatment for dystonia and PD, these side effects can reduce compliance. For this reason, it is important to focus attention on development of novel muscarinic agents that would selectively target specific receptor subtype, as there is still unmet clinical need. Table 1.5 shows antagonists and their selectivity at muscarinic mammalian (including human) receptors commonly used in the clinic, as well as a number of compounds that are used as research tools in order to specifically determine receptor subtype involved in particular disease.

Values for binding affinities (pKi) of the anticholinergics were obtained from the literature. pKi of benztropine, trihexyphenidyl, scopolamine, tropicamide, pirenzepine and AFDX-116 were obtained from Lazareno *et al.,* (1990). M1 binding sites were acquired from [^{3}H]Pirenzepine binding to rat cortex; whereas [^{3}H]N-methylscopolamine was labelled to the other sites – M2 binding was from rat heart; M3 binding was from rat submandibular glands; M4 binding was from rabbit lung (Lazareno *et al.,* 1990). pKi of darifenacin were determined using Chinese hamster ovary cell lines (CHO-K1) (Napier & Gupta, 2002) and membranes of Sf-9 cells (Ikeda *et al.,* 2001) expressing M1 – M5 receptors.

Affinity constants for PD102807 were obtained from CHO expressing human M1 – M5 receptors (Olianas & Onali, 1999).

For the selective M4 NBI-675 compound, binding affinity was measured by inhibition of radioligand binding to membranes from CHO cells expressing human M1 – M5 receptors (Neurocrine Bioscience, Inc.).

Table 1.5 Muscarinic antagonists affinity constants
Log affinity constant for pK_i values (Lazareno *et al.* (1990); Caulfield (1993); Olianas & Onali (1999); Ikeda *et al.,* (2001); Napier & Gupta (2002); Betz *et al.*, (2007); *Neurocrine Biosciences, Inc).

Muscarinic antagonist	pKi				
	M1	**M2**	**M3**	**M4**	**M5**
Trihexyphenidyl	9.13	7.86	8.53	8.71	
Benztropine	9.6	8.42	9.15	9.05	
Pirenzepine	7.8-8.5	6.3-6.7	6.7-7.1	7.1-8.1	6.2-7.1
Darifenacin	7.5-7.8	7.0-7.4	8.4-8.9	7.7-8.0	8.0-8.1
Tropicamide	7.18	7.3	7.42	7.85	
Scopolamine	9.73	8.85	9.74	9.03	
AFDX-116	5.9-6.7	6.7-7.3	5.8-6.4	6.2-6.4	5.5
PD102807	5.6	5.4	6.1	7.7	5.3
NBI-675*	6.63	7.72	6.67	9.52	5.75

1.3.2.2 Muscarinic receptors expression in the striatal circuity

All five subtypes of muscarinic receptors are located in the striatum showing different distribution and concentrations, therefore they are the main target in order of improving the symptoms of PD and dystonia.

M1 subtypes are mainly excitatory receptors expressed in the medium spiny neurons (MSN) of both direct and indirect basal ganglia pathways, positioned either extrasynaptically or on dendritic spine necks (Hersch *et al.*, 1994). Their activation reduces the activity of potassium channels thus increasing responsivity to glutamatergic stimulation (Ben-Ari *et al.*, 1992). In addition, they interact with adenosine A2a receptors in the direct pathway and activate signalling of DARP-32 in striatopallidial neurons. While on corticostriatal glutamatergic neurons they act as heteroreceptors and control release of glutamate (Bernard *et al.*, 1992; Alcantara *et al.*, 2001).

By contrast, there is a very low expression of M3 receptors in the striatum, mainly on dendrites of MSN (Hersch *et al.*, 1994). The exact role of the M3 receptor is unclear, but may be involved in the presynaptic control of drive to the SNc (Miller & Blaha, 2005). Thus, presynaptic M3 receptors serve to counter excessive excitation of nigral dopamine cell activity.

M5 receptor subtypes are also expressed in the striatum in low amounts, their role is not well understood but they may be found on the nigrostriatal dopaminergic terminals and could facilitate inhibition of dopamine release (Hersch *et al.*, 1994; Foster *et al.*, 2014).

Muscarinic M2 receptors are located on cholinergic interneurons (ChI) where they perform as inhibitory autoreceptors and in addition they act as presynaptic inhibitory heteroreceptors on corticostraital glutamatergic and dopaminergic nigrostriatal terminals (Hersch *et al.*, 1994; Bernard *et al.*, 1998; Mesulam, 1998; Piggott *et al.*, 2002).

High levels of inhibitory M4 receptors are found on the striatal MSN, where they are colocalised with D1 dopamine receptor on the direct pathway (Bernard *et al.*, 1992; Ince *et al.*, 1997; Eglen, 2006). They are present in cholinergic neurons and on presynaptic corticostriatal glutamatergic neurons, where they reduce release of glutamate (Bernard *et al.*, 1998). They are also expressed on dopaminergic terminals (Oldenburg & Ding, 2011).

1.3.3 Effect of acetylcholine on MSN, corticostriatal, nigrostriatal and GABAergic neurons

ACh exerts a direct effect on MSN via M1 and M4 receptors. The main effect of ACh is mediated through excitatory M1 receptors of the indirect pathway (Calabresi *et al.*, 2006). The striatal MSN are silent at "down" state due to the presence of K^+ currents, however, due to the inputs from cortical glutamatergic neurons, mediated by NMDA receptors they fire a burst of action potentials ("up" state) (Shen *et al.*, 2007). ACh causes a slow depolarization of the MSN of the indirect pathway via M1 receptors, thus inhibition of these K^+ currents, and enhancing the excitability of the striatal efferents. Additionally, activation of M1 receptors suppresses the L-type channels stopping influx of Ca^{2+} activating K^+ channels that would normally result in after hyperpolarisation (Shen *et al.*, 2007). The overall effect on ACh on the indirect

pathway is to enhance the effect of glutamate and oppose the effect of dopamine, and so promote movement.

ACh can also inhibit the MSN in the direct pathway by activation of postsynaptic inhibitory M4 receptors (Aosaki *et al.*, 2010; Oldenburg & Ding, 2011). This would oppose the stimulatory effect of glutamate and dopamine (via D1 receptors), thus reducing motor function.

ACh has an indirect effect on BG function via presynaptic neurotransmitter release regulation from cortical, nigral and local afferents. A tonic inhibition of corticostriatal glutamate release on MSN is triggered by ACh acting via presynaptic M2/M4 receptor. Activation of presynaptic nicotinic receptors at nigrostriatal terminals may also contribute to release of dopamine (Ding *et al.*, 2010). In addition, GABA release from terminals of fast spiking inhibitory interneurons is also under control ACh via inhibitory M4 and excitatory nicotinic receptors (Wang *et al.*, 2006) (Fig. 1.3). ACh act also indirectly on synapses through activing M1 receptors with the used of endocannabinoid system (Narushima *et al.*, 2007).

Clearly ACh plays an important role in the regulation of striatal output and hence the fine control of motor function mediated through the thalamocortical output. However, interactions with other neurotransmitters also help regulate the control of movement.

1.3.4 Dopamine-acetylcholine interaction

There is an interaction between DA and ACh in the striatum that further modulates the activity of the direct and indirect output pathways. Action of both dopamine, via D2 receptors, and acetylcholine, via M2/M4 receptors, triggers suppression of glutamate release from corticostriatal terminals and this results in a "down" state of the MSN. However, in the indirect pathway, the "up" state of the MSN is induced directly by ACh acting via M1 receptors, whereas in the direct pathway DA induces the "up" state by acting on D1 receptors (Pisani *et al.*, 2007; Benarroch, 2012). ACh acts via nicotinic receptors on DA terminals to stimulate the release of DA and DA has a direct effect on ChI, mainly by activation of D2 receptors, resulting in decrease of striatal ACh efflux and lessening autonomous action potential firing and synaptic input to ChI (Fig. 1.3) (Yan *et al.*, 1997; Aosaki *et al.*, 2010; Bonsi *et al.*, 2011).

Striatal acetylcholine is acting through different subtypes of cholinergic receptors, as

described in section 1.2.1.2, and evokes various effects, for instance controls release of dopamine (Fig. 1.3) (Smolders *et al.*, 1997; Lester *et al.*, 2010). Studies on knock-out mice showed that absence of M4 and M5 receptors significantly reduced release of striatal dopamine, whereas the lack of M1 and M2 receptors had no effect (Zhang *et al.*, 2002). Since the M4 receptors are present on cell bodies of MSN of the direct pathway, their activation inhibits release of GABA in the striatum causing reduction of $GABA_A$- mediated inhibition of release of dopamine from striatal nerve terminals (Ronken *et al.*, 1993; Lester *et al.*, 2010).

1.3.5 Cholinergic interneurons in disease

1.3.5.1 Dystonia

It is unknown how the anticholinergics work in dystonia, but studies show that they are important in modifications to the coupling of D2 receptor in the ChI in mutant mice overexpressing torsinA (transgenic DYT1 mouse model). Usually signalling of D2 receptor decrease interneuronal autonomous spiking, while in these transgenic mice, activation of D2 resulted in increased excitation and not reduction in the ChI (Pisani *et al.*, 2006). Based on the results, Pisani and colleagues (2006) showed an increased inhibitory coupling of D2 receptors to Cav2 Ca^{2+} channels regulating opening of Ca^{2+}-dependent K^+ channels after the spike (Pisani *et al.*, 2006). It has been suggested that regulator of G-protein signalling, RGS9 may have a role (Cabrera-Vera *et al.*, 2004). Stimulation of the D2 receptor caused increase in interneuron spiking and increased ACh release, which could give an explanation to the use of anticholinergics in dystonia (Pisani *et al.*, 2007).

1.3.5.2 Parkinson's disease

The reduction of motor activity in PD is a consequence of the loss of striatal dopaminergic innervation from the SNc, caused by increased activity of basal ganglia output nuclei to the motor cortex (see above). DA depletion, as seen in PD, results in an increase in cholinergic activity, and, although the cholinergic interneurons are rich in muscarinic autoreceptors which employ a feedback control on ACh release, the interneurons remain hyperactive.

Cholinergic interneurons express high levels of D2 receptors, which decrease release of ACh by weakening the Cav2 Ca^{2+} channels in response to membrane depolarization

(Yan *et al.*, 1997; Ding *et al.*, 2006). In addition, it has been suggested that a decrease in the M4 autoreceptor efficacy, which is a consequence of DA depletion, contributes to this increase in ACh signaling (Ding *et al.*, 2006; Pisani *et al.*, 2007).

Possibly more importantly, ACh subsequently acts at muscarinic M1 and M4 receptors on MSN output neurons, validating the use of anticholinergics in the treatment of PD. Non-selective anticholinergics are used to treat PD, however, targeting the cholinergic neurons specifically located in the striatum may still alleviate the motor symptoms but with reduced side effects. In particular, the M4 muscarinic receptor is found in abundance in the striatum, and not in the periphery, where they have inhibitory action on D1 receptor-mediated locomotor stimulation (Gomeza *et al.*, 1999a). Therefore, we propose that inhibition of muscarinic M4 receptors may disinhibit the direct pathway, and potentially enhance its activity in the presence of dopamine, for example following treatment with L-DOPA.

Currently used anticholinergics are not particularly selective for the specific subtypes of muscarinic receptors (Table 1.5), and the lack of subtype-specific compounds makes it difficult to understand the involvement of exact muscarinic subtype in control of movement, without inducing unfavorable, bothersome side effects associated with the use of the non-selective anticholinergics, including dry mouth, cognitive impairment or blurred vision. The highly selective muscarinic M4 antagonists appears as a good as a promising therapeutic approach, therefore the studies described in this thesis would assess the effect of the novel highly selective muscarinic M4 antagonist, NBI-675, and compare its effect with the currently used anticholinergics on motor control using animal models of dystonia and PD.

1.4 Thesis hypothesis

Anticholinergics are commonly used in the treatment of dystonia and Parkinson's disease but currently used anticholinergics are non-selective for different subtypes of muscarinic receptors located both centrally and peripherally, therefore, their use is limited due to unwanted side effects. There is a strong evidence suggesting that muscarinic M1 and M4 receptors, which are highly abundant in the striatum, are associated with the control of involuntary movements. Since the M4 receptors are largely located in this part of the brain, they are a target for the treatment with minimised side effects profile. Thus, it is hypothesised that muscarinic M4 selective

antagonists control abnormal involuntary movements and motor deficits in dystonia and Parkinson's disease with reduced side effect profile.

1.5 Thesis aims

In order to test the hypothesis, the studies described in this thesis investigated the role of muscarinic antagonists (anticholinergics), and particularly a novel selective muscarinic M4 antagonist, NBI-675, as a potential treatment in reduction of involuntary movements, without inducing peripheral side effects, such as oral dryness, in established *in vivo* animal models.

Specifically, the following aims were addressed:

1) To determine if selective antagonism of muscarinic M4 receptors controls dystonic movements with reduced side effects profile;
2) To determine if selective antagonism of M4 muscarinic receptors reverses motor disability in MPTP-treated common marmoset model of Parkinson's disease;
3) To determine if selective antagonism of M4 muscarinic receptors reverses dyskinesia in MPTP-treated common marmoset model Parkinson's disease.

Chapter 2 Materials and Methods

2.1 Introduction

The aim of the studies reported in this thesis was to investigate the central and peripheral activity of anticholinergics of different relative selectivity for muscarinic receptors using *in vivo* animal models with a view of improving the treatment of dystonia and Parkinson's disease with the use of the selective muscarinic M4 antagonists.

2.2 Experimental animal models

Animal models play important role in the research, as they not only give insights into the underlying mechanisms, but also help to improve existing treatment and contribute to explore different avenues for therapeutic development. In an ideal world, animal models should present all the relevant symptoms and pathology seen in humans, however, this is rarely the case. Since it is hard to model every aspect of the disease, particularly motor function and disability such as PD, dystonia and dyskinesia, as they are very heterogeneous conditions, animal models should be tailored to the type of the disease and requirements from the model. Currently, there are number of different animal models that display features associated with particular line of investigation, including genetic models, such as transgenic or knock-out rodent models, for example mouse models expressing mutant proteins involved in familial PD (Goldberg *et al.*, 2003) or *Cacna1a* knock-out mouse model of generalised dystonia (Jinnah *et al.*, 2005). Furthermore, toxin-induced models are broadly used in research, as they can be useful in evaluating new or improved treatments. To date, there is no ideal model to reflect dystonia seen in man, however, numerous toxin-induced animal models are extensively employed to investigate motor complications and abnormal movements, such as 6-hydroxydopamine (6-OHDA)-lesioned rodent model and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated primate, which are considered as the gold standard models, since they reliably reproduce motor complications seen in man (Cenci & Ohlin, 2009; Duty & Jenner, 2011). Furthermore, there is a lack of models that could be utilised to investigate the cholinergic component involved in PD and dystonia, nevertheless the use of cholinergic agonists has been reported to induce involuntary oral movements in rats, resembling chewing, thus this model together with the MPTP-treated primate model provides useful and excellent tool in the assessment of novel treatments with potential antiparkinsonian and antidystonic properties

(Salamone *et al.*, 1986; Stewart *et al.*, 1988). For this purpose, the pilocarpine-induced purposeless chewing movement model in rat was established. In addition, motor disability and dyskinesia with dystonia and chorea was assessed in the MPTP-treated common marmoset. These models were specifically used to probe the process of expression of dystonia and its inhibition by anticholinergic agents, including a selective M4 antagonist. Drug-induced expression of dystonia was assessed by using a manual behavioural scoring system. Peripheral side effect profile of anticholinergics was investigated by measuring M3-mediated saliva production in rats. The general methodologies employed in these studies are described in detail below.

2.2.1 Pilocarpine-induced purposeless chewing model in rats

Substantial evidence indicates that imbalance between cholinergic and dopaminergic system is involved in formation of involuntary movements (Duvoisin, 1967) and hence, anticholinergics have been used in the treatment of both PD and dystonia since decades. Studies on rodents, particularly on rats, demonstrate that administration of cholinomimetic agents provoke involuntary movements in the form of oral movements of the lower jaw, which resemble chewing and are a result of central stimulation of muscarinic receptors located in the striatum (Salamone *et al.*, 1986; Stewart *et al.*, 1989; Finn *et al.*, 1997; Mayorga *et al.*, 1997). Furthermore, systemic administration of a non-selective cholinergic agonist, pilocarpine, acts both centrally and peripherally on muscarinic receptors, causing series of effects, including increased salivation, lachrymation, urination, diarrhoea, piloerection. These effects can be reversed by anticholinergics (Stewart *et al.*, 1989; Mayorga *et al.*, 1999). Previous studies have indicated that the vertical movements of the jaw are caused by stimulation of centrally located muscarinic receptors (Stewart *et al.*, 1988; Mayorga *et al.*, 1999). Therefore, the model of pilocarpine-induced chewing has been used previously to assess the cholinergic component in the formation of involuntary movements. Since this effect is mediated centrally, previous studies have suggested that M4 receptor are responsible for this motor activity (Mayorga *et al.*, 1999; Betz *et al.*, 2007), hence this model may provide important information about the central cholinergic function and its involvement in production of involuntary movements, such as dystonia.

2.2.1.1 Rat

Male Wistar rats (150 – 350 grams, Harlan, Bicester, UK) were used in all rat procedures. Animals were housed in groups of 2 – 4 per cage in a temperature (22 ± 2°C) and humidity controlled housing unit with 12 hours of light and dark. Nesting material and cardboard pipes were provided as a form of enrichment. Food and water was available *ad libitum*, except during the time of behavioural testing. Rats were habituated to the testing environment at least one week prior testing, and had 30 min of acclimation to testing room before the experiment. All efforts were made to minimize the number of animals used and their suffering. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 under UK Home Office Project licence number 70/6898 and 70/7977 approved by the King's College London Ethical Review Panel.

2.2.1.2 Assessment of chewing behaviour in response to increasing doses of pilocarpine

This method was adapted and modified from Stewart *et al.* (1988). Animals were placed in a Perspex observation boxes (21 x 35 x 17 cm) with mirrors placed behind (Figure 2.1), to allow for the viewing of animal from several angles.

Figure 2.1 Perspex boxes set up for measuring purposeless chewing behaviour in rats. Mirrors are placed behind cages to allow for the viewing of animals from several angles as animals move around.

The number of purposeless chewing movements was recorded for 1 min every 10 min using a mechanical hand counter by a trained observer. Chewing behaviour was

characterised as rapid and repetitive jaw movements, which were not directed at any particular object or stimulus (Salamone *et al.*, 1986; Stewart *et al.*, 1988). Counting was halted if an animal groomed, yawned or bit during the observation, until 5 s after the termination of activity (Stewart *et al.*, 1988).

2.2.1.3 Statistical analysis

Data and statistical analysis were performed using GraphPad Prism 5.02 (San Diego, CA, USA). Area under curve (AUC) for the time courses was calculated by trapezoid method. Data are expressed as mean ± SEM and were analysed by sigmoidal nonlinear regression analysis 4-parameter fit. ED_{50} value was derived from the curve fit. Differences between treatment and vehicle control for AUC data was analysed by non-parametric Friedman's followed by *post hoc* Dunn's test.

2.2.1.4 Results of the assessment of purposeless chewing behaviour induced by pilocarpine

Animals were placed in a Perspex observation boxes and basal assessment was conducted. Pilocarpine (0.1 – 32 mg/kg), a non-selective cholinergic agonist, or vehicle were administered intraperitoneally (i.p.) in a randomised manner according to a modified Latin square design, to a group of six rats. Purposeless chewing assessment started 10 min post pilocarpine administration as described in section 2.2.3.1. Systemic administration of pilocarpine (0.1 – 32 mg/kg i.p.) produced rapid up and down jaw movements. These purposeless chewing-like movements were visible after administration of all doses of pilocarpine within 10 min of drug administration, with maximal response between 20 and 30 min post-injection (Fig. 2.2 A). The present work confirms previous findings (Salamone *et al.*, 1986; Stewart *et al.*, 1988; Stewart *et al.*, 1989; Mayorga *et al.*, 1999; Betz *et al.*, 2007) that purposeless chewing behaviour in rats can be induced by systemic administration pilocarpine.

The effect of pilocarpine-induced chewing was dose-dependent and it was significantly different for the four higher doses (4 – 32 mg/kg) when compared to vehicle (Fig. 2.2 B). The ED_{50} in the current study was 3.4 mg/kg (Fig. 2.2 B) and agrees with previously published data, where a dose of 4 mg/kg was used to induce chewing behaviour (Stewart *et al.*, 1988; Stewart *et al.*, 1989; Mayorga *et al.*, 1999; Betz *et al.*, 2007). Consequently, based on the results of this study, a single dose of

pilocarpine (3.4 mg/kg i.p.) was selected for use in the remaining experiments, where anticholinergic agents were tested (Chapter 3).

Along with induction of rapid chewing movements, administration of pilocarpine resulted in other visible manifestations, such as excessive piloerection, lacrimation, salivation, increased urination and defecation. This is also consistent with studies published in the literature (Stewart *et al.*, 1988; Mayorga *et al.*, 1999; Betz *et al.*, 2007), and is the result of stimulation of peripherally located muscarinic receptors in the skin (M1), salivary glands (M1 and M3), bladder (M3) and gut (M3) (Eglen, 2006; Langmead *et al.*, 2008).

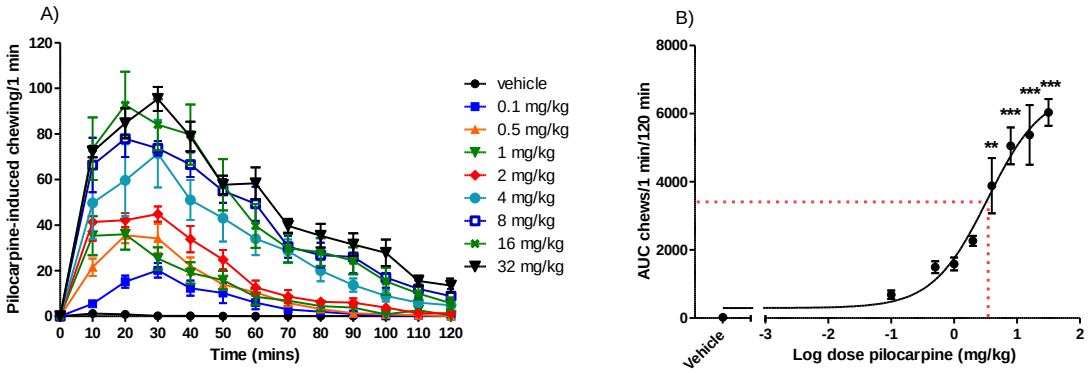


Figure 2.2 Pilocarpine-induced purposeless chewing behaviour in rats.
Pilocarpine 0.1 – 32 mg/kg i.p. A) Time course. Data are mean ± SEM (n=6); B) Log-dose chewing (AUC of time course). Data were analysed by a non-linear curve fit (variable slope). ED_{50} = 3.4 ± 1.5 mg/kg (dotted line); (95% CI = 1.48 – 7.89 mg/kg); r^2 = 0.78 $p<0.05$; **$p<0.01$; ***$p<0.001$ compared to vehicle (Friedman's test followed by post hoc Dunn's test).

2.2.2 Measurement of salivation as an index of peripheral muscarinic activity

Administration of anticholinergics, in treatment of dystonia or Parkinsonism, causes countless central and peripheral side effects (Chapter 1, section 1.3.2.1), in particular oral dryness (Goldman & Comella, 2003; Adam & Jankovic, 2007) due to their inhibitory effect on muscarinic receptors.

To confirm whether selective muscarinic M4 receptor antagonists are a viable treatment for dystonia without associated peripheral side effects, measurements of saliva secretion in rats was conducted. Pilocarpine, as a non-selective cholinergic

agonist, stimulates saliva secretion by acting directly on M3 acetylcholine receptors located in the salivary glands (Eglen, 2006; Langmead *et al.*, 2008).

2.2.2.1 Methods of measurement of salivation

This method was adapted and modified from Flynn *et al.* (1980). Briefly, a 15 cm cotton tipped swab (Johnson's & Johnson) was weighed. By holding an animal in non-dominant (left) hand, the swab was inserted into the mouth (usually left cheek side) and kept there for a period of 10 sec. The swab was immediately reweighed to measure the weight of saliva, which was calculated by subtracting the initial from final weight of the swab (Flynn *et al.*, 1980).

2.2.2.2 Statistical analysis

Data and statistical analysis were performed using GraphPad Prism 5.02 (San Diego, CA, USA). Area under curve (AUC) for the time courses was calculated by trapezoid method. Data are expressed as mean ± SEM and were analysed by sigmoidal nonlinear regression analysis 3-parameter fit. ED_{50} value was derived from the curve fit. Differences between treatment and vehicle control for AUC data was analysed by One-way ANOVA followed by *post hoc* Dunnett's test.

2.2.2.3 Results of the assessment of pilocarpine-induced saliva secretion

Pilocarpine (0.5 – 8 mg/kg i.p.) or vehicle were administered in a randomised manner according to a modified Latin square design to a group of five rats and saliva production was measured before (time 0) and every 10 min for 40 min after injection of pilocarpine, as described in section 2.2.2.1. Administration of increasing doses of pilocarpine (0.5 – 8 mg/kg) produced a dose-related increase in saliva secretion in rats. Excessive saliva production was visible within 3 – 5 min, with maximal response 10 min after drug administration (Fig. 2.3 A). The effect was significant for doses 0.5 – 8 mg/kg when compared to vehicle treated animals (Fig. 2.3 B). The ED_{50} was 2.65 mg/kg, however, for the consistence of the studies, an ED_{50} dose obtained from study on purposeless chewing behaviour (section 2.2.1.4) of 3.4 mg/kg was chosen to be used in further analysis (Chapter 4).

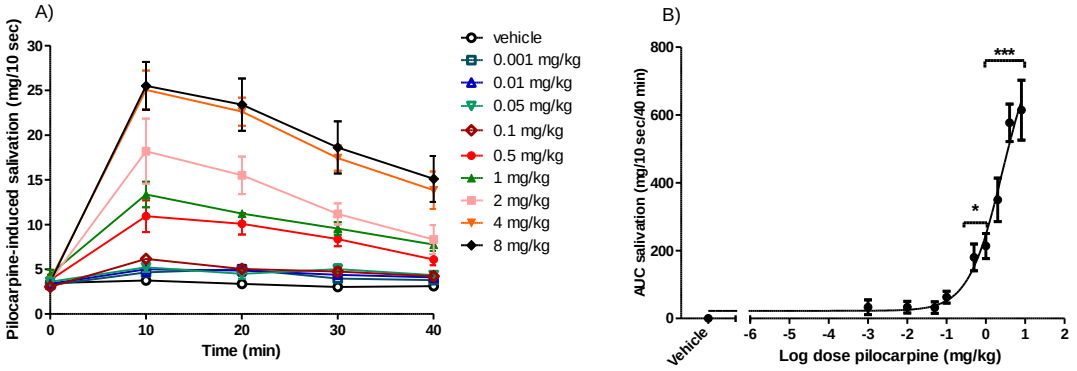


Figure 2.3 Pilocarpine-induced saliva secretion in rats.
Pilocarpine (0.001 – 8 mg/kg i.p.) A) Time course. Data are mean ± SEM (n=5); B) Log dose-related induction of saliva secretion (mg/10 sec/40 min) (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values using GraphPad Prism. ED_{50} = 2.65 ± 1.38 mg/kg; (95% CI = 1.38 – 5.09 mg/kg); r^2 = 0.85 $p<0.05$; *$p<0.05$; ***$p<0.001$ compared to vehicle (One-way ANOVA followed by post hoc Dunnett's test).

2.2.3 The MPTP-treated primate model

The parkinsonian-like symptoms of MPTP were discovered in 1908s following the unintentional administration of contaminated street drugs. This event led to the major changes in the research and modelling of PD (Langston *et al.*, 1984).

MPTP crosses the BBB and becomes oxidised by MAO-B to its active metabolite (1-methyl-4-phenylpyridinium (MPP^+), which inhibits mitochondrial respiratory chain Complex I, leading to formation of cytotoxic free radicals (Smeyne & Jackson-Lewis, 2005). Systemic administration of MPTP results in degeneration of dopaminergic neurons in the SN (Iravani *et al.*, 2005) triggering appearance of motor symptoms, including bradykinesia, rigidity and postural abnormalities (Jenner *et al.*, 1984). Humans, non-human primates and some strains of mice are particularly susceptible to the effects of the MPTP, however, rats remain resistant to the effects of the toxin. It is thought that possibly due to their high capacity for vesicular sequestration of MPTP. The toxin found the usefulness in modelling PD in non-human primates, particularly behavioural symptoms have been characterised in cynamologous monkeys (*Macaca fascicularis*), common marmosets (*Callithrix jacchus*), squirrel and rhesus monkeys (Jenner *et al.*, 1984).

Non-human primates treated with MPTP respond well to L-DOPA and dopaminergic drugs. Moreover, as seen in humans, chronic administration of L-DOPA results in a development of dyskinesia with the manifestation of chorea, dystonia and athetosis, closely resembling those seen in PD patients (Jenner, 2003b). Furthermore, "on-off" and "end of dose deterioration" occurrences and rebound worsening are also exhibited (Pearce *et al.*, 1995; Kuoppamaki *et al.*, 2002) therefore, the model provides high predictability in assessing novel treatment and in translating preclinical drug studies into the clinical setting (Kuoppamaki *et al.*, 2007). Nevertheless, the limitation of the model is absence of progressive pathology and prompt development of dyskinesia (Langston *et al.*, 1984). Administration of MPTP results mainly in nigrostriatal degeneration but lesions and cell loss were also reported in hypothalamus and locus coeruleus (Langston *et al.*, 1984; Crossman *et al.*, 1985; Gibb *et al.*, 1986). The loss of dopaminergic neurons is not progressive and Lewy bodies are not exhibited in MPTP-treated primates (Forno *et al.*, 1993; Kowall *et al.*, 2000).

2.2.3.1 Animals

Adult common marmosets (*Callithrax Jacchus*) (Harlan, UK, 350 – 500 g, n = 6-8 per group) of either sex were used in this study. Animals were previously treated with MPTP and primed with L-DOPA and were not drug naïve prior to the study.

2.2.3.1.1 Animal husbandry

Upon arrival animals were given at least two weeks to acclimatise to their new environment. During this period, animals were assessed for general health. Animals were housed singly or in pairs (mixed ♂♀or single sex ♀♀) in controlled environment suitable for the species (temperature 25 ± 1° with 50% relative humidity on a 12 hour light/dark cycle). All animals had *ad libitum* access to water and Mazuri food pellets (Mazuri Primate Diet, Special Diet Services Ltd., UK) and received two meals each day, mashed up Mazuri pellets, forage mix and pumpkin seeds in the morning, and fresh fruits in the afternoon. All experiments were carried out in accordance with Home Office regulations under the Animals (Scientific Procedures) Act 1986 and project licence number 70/7146 and 70/8541.

2.2.3.2 MPTP-lesion induction

Between 2.5 – 5.5 years prior to these studies common marmosets were treated with MPTP in order to induce the motor symptoms of PD according to previously established protocols (Pearce *et al.*, 1995). Animals received subcutaneous injections of MPTP (2 mg/kg) (Sigma, UK/Research Biochemicals International) in sterile saline 0.9% daily for 5 consecutive days. Within a few days following MPTP treatment, animals exhibited symptoms that resemble Parkinson's disease, including bradykinesia, akinesia and rigidity, along with loss of vocalisation, diminished blinking and action tremor. Animals initially became unable to feed or take care of themselves, therefore were hand fed up to twice a day every day with marmoset jelly/liquid diet until they were able to take care of themselves and their body weight had stabilised (8 – 10 weeks) (Pearce *et al.*, 1995; Goula *et al.*, 2012). Animals recovered gradually and at this stage behavioural assessment indicated all animals had similar stable level of motor deficits. MPTP treatment leads to a syndrome where behavioural deficits remain stable over the years (Jenner *et al.*, 1984).

MPTP treatment and subsequent animal husbandry were performed by Michael Jackson and Ria Fisher.

2.2.3.2.1 L-DOPA priming for dyskinesia

L-DOPA priming had been carried out prior to this study in order to establish stable and reproducible expression of dyskinesia in animals. Following recovery from acute effects of MPTP treatment, animals were primed by the daily administration of L-DOPA methyl ester (8 mg/kg p.o.) + benserazide (10 mg/kg p.o.) twice daily for 5 days a week (5 days of treatment followed by a period of 2 days with no treatment), for up to 30 days. During this period, animals progressively developed dyskinesia with increasing severity, which eventually remained stable. The overall effect of L-DOPA resulted in an increase of locomotor activity, reversal of motor disability and expression of moderate to severe dyskinesia.

L-DOPA priming and assessment were performed by Michael Jackson and Ria Fisher.

2.2.3.2.2 L-DOPA challenge and animal selection

The animals were used in previous studies and were not drug naïve. Following the priming period, subsequent administration of L-DOPA elicits the same dyskinetic response for subsequent studies.

Prior to studying the effects of anticholinergics in MPTP-treated L-DOPA-primed marmosets, animals (n = 12) were re-challenged with L-DOPA (8 mg/kg p.o.) + benserazide (10 mg/kg p.o.) to ensure a good motor response and dyskinesia expression. Animals were placed in the automated test cages fitted with photo beams, as described in section 2.2.3.3. Locomotor activity and reversal of motor disability and dyskinesia (dystonia and chorea) were assessed as described in section 2.2.3.3.

The animals demonstrating best overall L-DOPA response, i.e. increase in locomotor activity and dyskinesia (dystonia and chorea) and reversal of motor disability were used in subsequent experiments. The response to L-DOPA administration was visible within few minutes of dosing the drug, and it was manifested by improvement of locomotor activity (Fig. 2.4 A) and reversal of motor disability (Fig. 2.4 B) with the appearance of dyskinesia which manifested as chorea and dystonia (Fig. 2.4 C). Based on the obtained results, the eight animals which produced the greatest overall response to L-DOPA (improvement in locomotor activity, expression of dyskinesia and reversal of motor disability) were chosen for further studies.

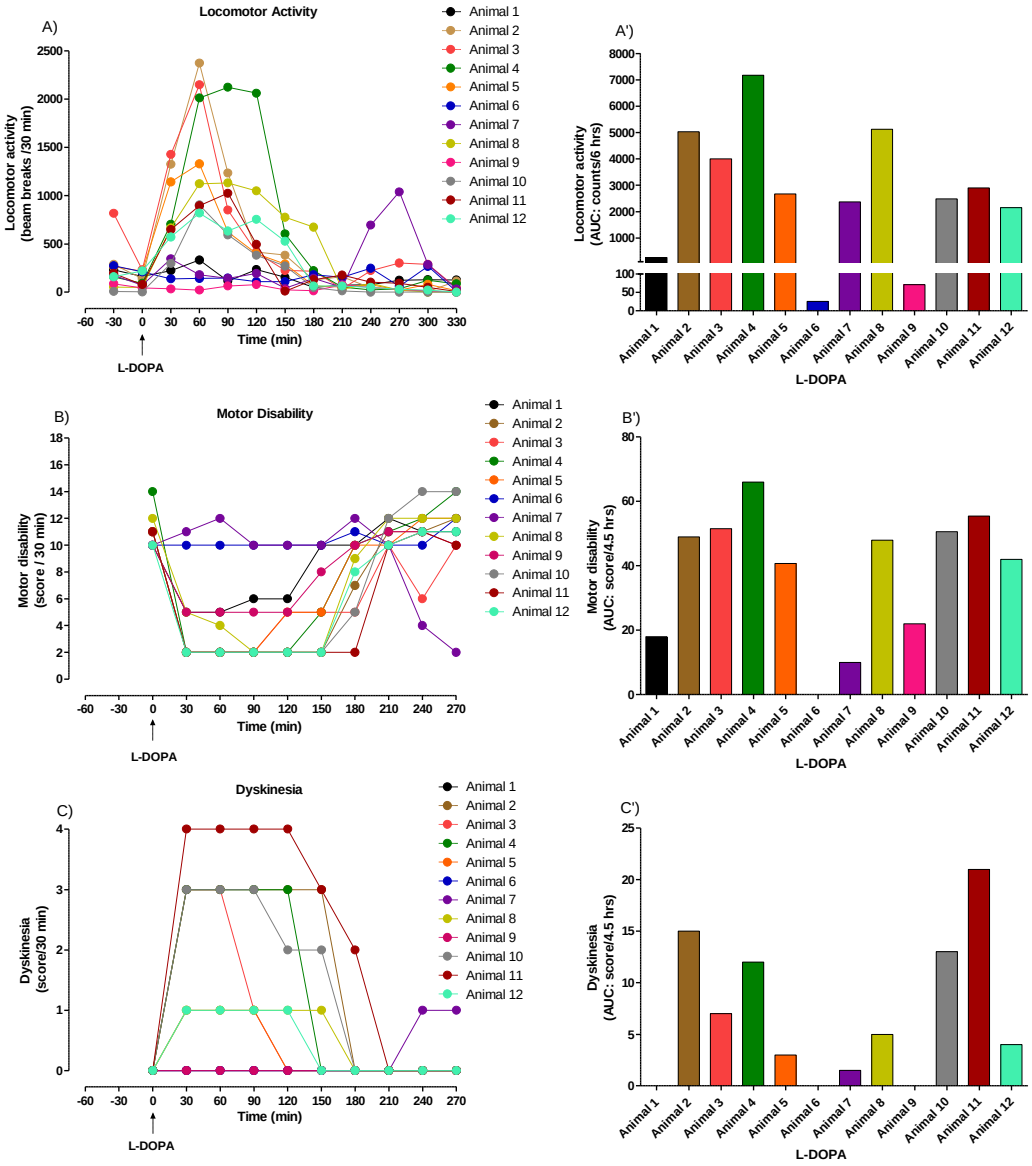


Figure 2.4 The effect of L-DOPA on locomotor activity, motor disability and dyskinesia in MPTP-treated common marmosets

Time course effect on A) Locomotor Activity; B) Motor Disability and C) Dyskinesia; AUC of time course A') Locomotor Activity; B') Motor Activity and C') Dyskinesia. (n = 12). Animals were placed into the test units and baseline behaviour assessed. After 1 hour (t = 0) animals were treated with L-DOPA (8 mg/kg p.o. + benserazide 10 mg/kg p.o.) and behavioural assessment was conducted for further 4.5 hours. Animals which showed the best overall increase in locomotor activity, reversal of motor disability and expression of dyskinesia were selected for further studies.

2.2.3.3 Behavioural assessment

All behavioural assessments were carried out between the hours 7.00 am and 3.00 pm. The assessment consisted of examining and scoring locomotor activity, motor disability reversal and dyskinesia (chorea, dystonia and overall dyskinesia) in specially designed aluminium test units (50 x 60 x70 cm) with Perspex doors (50 x 70 cm), fitted with photocells to measure locomotor activity (Fig. 2.5). Animals were given 60 min of acclimatization, during which a baseline score was determined (Smith *et al.*, 2003). Test units were housed in a behaviour test room and animals were observed by experienced observers blinded to the drug treatment through one-way mirror, as described below.

2.2.3.3.1 Locomotor activity assessment

Each test unit was fitted with eight horizontally orientated infrared beams to detect movement of the animals (Fig. 2.5 B). Beam interruptions were automatically recorded as a single locomotor count and transmitted to the computer software (DASYLab data acquisition system, laboratory version 11 and a software custom made by Dave Leyman using Activity Monitor National Instruments CVI Windows platform) throughout the test period and accumulated in 1 min intervals. The data was plotted as total counts per 30 min periods for the 7 hrs experiment duration to produce a time course of the drug activity (Smith *et al.*, 2003). From the time course data, the AUC (GraphPad Prism) was calculated and presented as a total locomotor activity over duration of experiment.

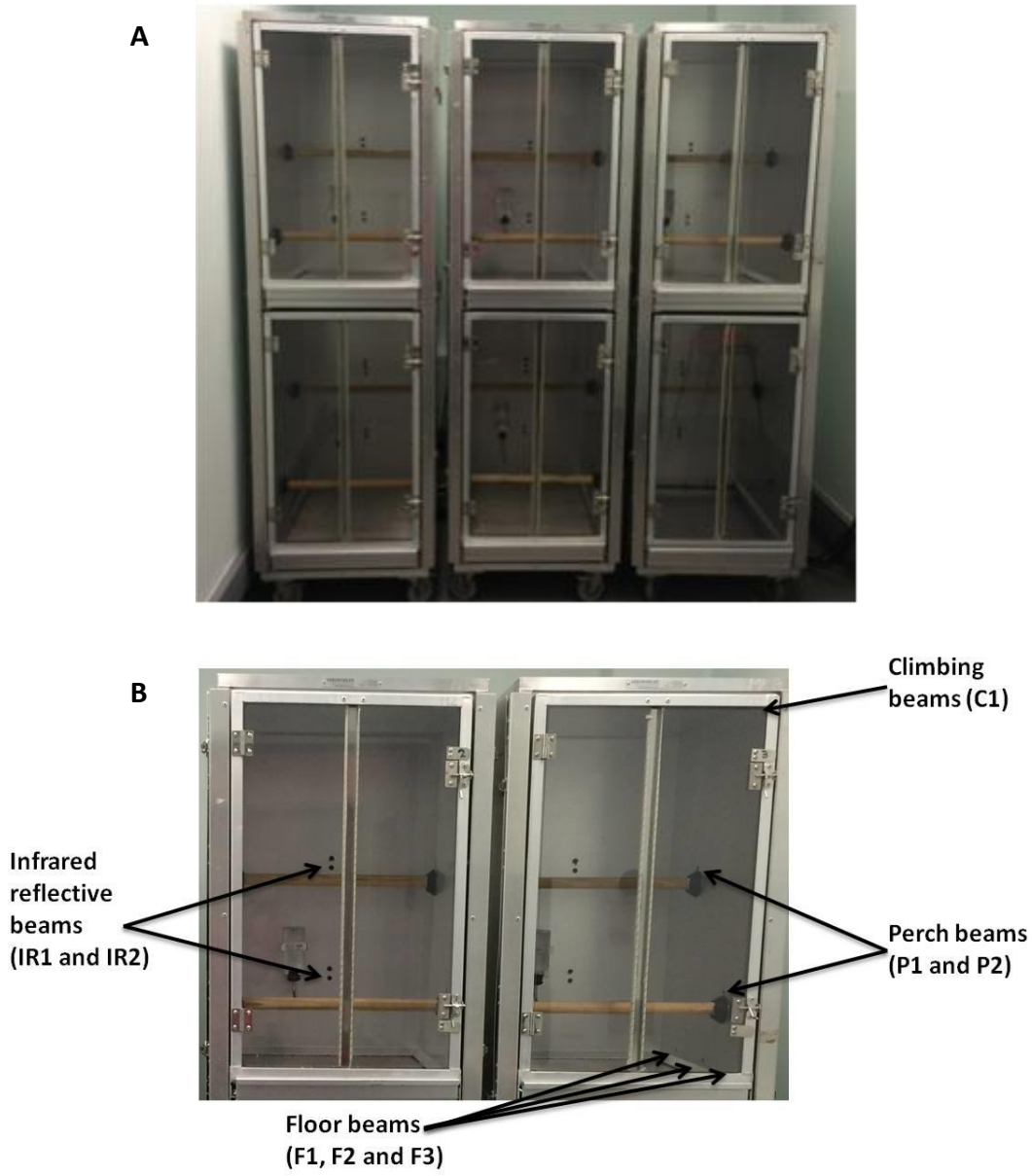


Figure 2.5 Marmosets test units (A) and the position of infrared beams detecting movement (B).

2.2.3.3.2 Motor disability assessment

Motor disability was scored simultaneously with locomotor activity measurement through one-way mirror during the last 10 min of each consecutive 30 min time intervals. Baseline scores were being collected at the end of acclimatisation period, at 50 – 60 min after animals had been placed into the test units, prior to the drug treatment. The assessment of motor disability scoring was based on previously established motor disability scale, as shown in Table 2.1 (Pearce *et al.*, 1995). Total

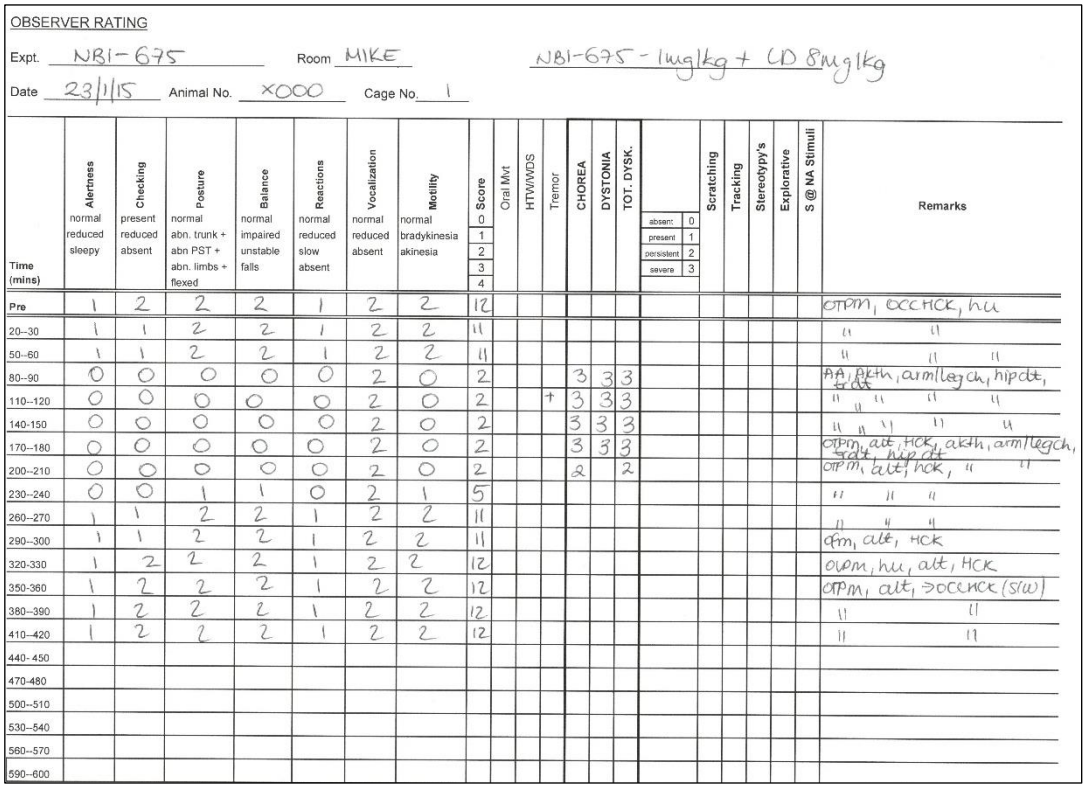
score consisted of sub-scores given to each of individual criterion. A maximum possible score for motor disability was 18, which indicates a severely disabled marmoset, however, MPTP-treated marmosets usually have score of 10 – 14 without the drug treatment, while normal naïve marmosets usually score 0 for motor disability. Additional behavioural observations, including stereotypy, vomiting, scratching, tracking or staring at non-apparent stimuli, explorative behaviour, repetitive movements (i.e. head checks in the same direction or circling cage floor with jumps) were recorded as additional notes, which were not used for analysis, but were used qualitatively to help to determine overall effect of the drug tested. A typical example of score sheet for motor disability is shown in Figure 2.6.

Table 2.1 Motor Disability and dyskinesia scoring scale.
Adapted from Pearce *et al.* (1995).

Severity						
		0	**1**	**2**	**3**	**4**
Disability scores	**Alertness**	Normal	Reduced	Sleepy		
	Checking movements	Normal	Reduced	Absent		
	Posture	Normal	One from: Abnormal trunk/limbs/ tail	Two from: Abnormal trunk/limbs/ tail	Abnormal trunk, limbs and tail	Flexed – grossly abnormal
	Balance/ coordination	Normal	Impaired	Unstable	Spontaneous falls	
	Reactions	Normal	Reduced	Slow	Absent	
	Vocalisation	Normal	Reduced	Absent		
	Motility	Normal	Bradykinesia /reduced	Akinesia/ absent		
Dyskinesia scores	**Scored separately for dystonia, chorea and overall dyskinesia**	Absent	Mild, fleeting and rare dyskinetic postures and movements	Moderate, more prominent abnormal movements but not significantly affecting normal behaviour	Marked, frequent and at times continuous dyskinesia affecting normal pattern of activity	Severe, virtually continuous dyskinetic activity disabling to animal and replacing normal behaviour

2.2.3.3.3 Dyskinesia assessment

Dyskinesia was assessed at the same time as motor disability, using previously established dyskinesia rating scale as shown in Table 2.1 (Pearce *et al.*, 1995). Animals were also scored separately for chorea and dystonia. Chorea manifests as rapid, dance-like, flicking movements predominantly of the limbs and head; whereas dystonia is characterised by abnormal sustained movements and postures, encompassing mainly arm, leg and trunk. Other features that are included in overall dyskinesia score include athetosis (sinuous writhing limb movements) and akathisia (motor limb restlessness, piano playing-like movements) (Pearce *et al.*, 1995). The score recorded for these three parameters reflects the quality and quantity of dyskinetic activity over the 10 min observation period. A typical example of disability score sheet is shown in Figure 2.6.



OBSERVER RATING

Expt. NBI-675 Room MIKE NBI-675 - 1mg/kg + LD 8mg/kg

Date 23/1/15 Animal No. X000 Cage No. 1

Time (mins)	Alertness: normal / reduced / sleepy	Checking: present / reduced / absent	Posture: normal / abn. trunk + abn PST + abn. limbs + flexed	Balance: normal / impaired unstable / falls	Reactions: normal / reduced slow / absent	Vocalization: normal / reduced / absent	Motility: normal / bradykinesia / akinesia	Score: 0 / 1 / 2 / 3 / 4	Oral Mvt	HTW/WDS	Tremor	CHOREA	DYSTONIA	TOT. DYSK.	absent: 0 / present: 1 / persistent: 2 / severe: 3	Scratching	Tracking	Stereotypy's	Explorative	S @ NA Stimuli	Remarks
Pre	1	2	2	2	1	2	2	12													OPPM, OCCHCK, hu
20–30	1	1	2	2	1	2	2	11													" "
50–60	1	1	2	2	1	2	2	11													" " "
80–90	0	0	0	0	0	2	0	2				3	3	3							AA, akth, armlegch, hipdt, tdt
110–120	0	0	0	0	0	2	0	2			+	3	3	3							" " " "
140–150	0	0	0	0	0	2	0	2				3	3	3							" " " "
170–180	0	0	0	0	0	2	0	2				3	3	3							OPPM, alt, HCK, akth, armlegch, tdt, hip dt
200–210	0	0	0	0	0	2	0	2				2		2							OPPM, alt, hck, " "
230–240	0	0	1	1	0	2	1	5													" " "
260–270	1	1	2	2	1	2	2	11													" " "
290–300	1	1	2	2	1	2	2	11													OPM, alt, HCK
320–330	1	2	2	2	1	2	2	12													OPM, hu, alt, HCK
350–360	1	2	2	2	1	2	2	12													OPPM, alt, ⇒OCCHCK (S/W)
380–390	1	2	2	2	1	2	2	12													" "
410–420	1	2	2	2	1	2	2	12													" "
440–450																					
470–480																					
500–510																					
530–540																					
560–570																					
590–600																					

Figure 2.6 An example of the score sheet for motor disability and dyskinesia.
Assessment following treatment with NBI-675 (1 mg/kg p.o.) + L-DOPA (8 mg/kg p.o. + benserazide (10 mg/kg p.o.)).

2.2.3.4 Drug treatment

Benztropine mesylate (0.25 & 0.5 mg/kg), scopolamine hydrobromide (0.1 & 0.3 mg/kg) and methylscopolamine (0.1 & 0.3 mg/kg) were dissolved in 0.9% saline and administered subcutaneously (s.c.) at the dose volume 1 ml/kg. DL-Trihexyphenidyl hydrochloride (0.5 & 1 mg/kg) was dissolved in 10% sucrose made up in deionised water and administered by oral gavage (p.o.). NBI-675 (1, 5 & 7.5 mg/kg) was dissolved in 5% Tween80 and 0.5% methylcellulose made up in deionised water, vortexed and sonicated for 1 hr. The pH was adjusted to 4 – 6 with 1M NaOH. NBI-675 was administered by oral gavage (p.o.). Drugs administered orally were given at a dose volume of 2 ml/kg.

All anticholinergics were administered 1 hour prior L-DOPA (8 mg/kg p.o.) + benserazide (10 mg/kg p.o.) in 10% sucrose. Doses of the drugs were chosen according to the previously published studies (Jackson *et al*., 2013) and according to information based on the PK and PD studies (NBI-675) received from Neurocrine Biosciences Inc.

Animals were placed into the testing units. Following the 60 min acclimatisation period which provided baseline activity data, animals were dosed with appropriate anticholinergic or vehicle followed by L-DOPA (8 mg/kg p.o.) + benserazide (10 mg/kg p.o.) 60 min later. Behavioural assessment was then carried out as described in Section 2.2.3.3. A repeated crossover design was used, so each marmoset was treated with all doses of a single drug or vehicle with at least 72 hrs washout between the tests. A typical modified Latin square was used to randomise drug treatments and is shown in Table 2.2.

Locomotor activity was recorded throughout the study. Motor disability and dyskinesia were scored immediately before each drug treatment and then every 30 minutes for 10 minute periods throughout the study for up to 5 hours, as shown in Figure 2.7.

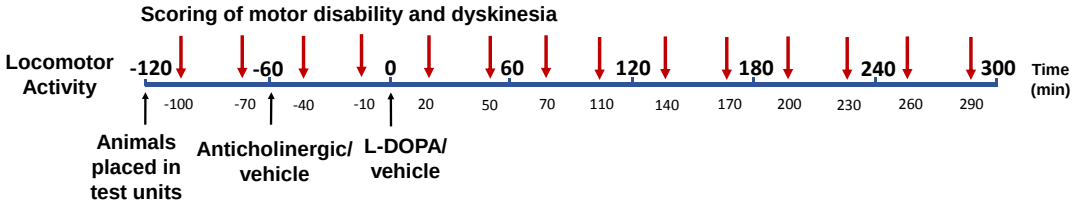


Figure 2.7 Timeline presenting the study design.
Animals were placed into the test units at t=-120 min and were given 60 min of habituation before the anticholinergic treatment t=-60 min, followed by L-DOPA 60 min later (t=0 min). Locomotor activity was recorded throughout the study. Motor disability and dyskinesia were scored immediately before each drug treatment and then every 30 minutes for 10 minute periods throughout the study for up to 5 hours (red arrows).

Table 2.2 Typical example of a latin square with a crossover designed treatment for administration of different doses of anticholinergics prior L-DOPA.

Animal number	Treatment day					
	1	2	3	4	5	6
1	A	B	C	D	E	F
2	D	A	E	B	F	C
3	C	F	A	E	D	B
4	B	D	F	A	C	E
5	E	C	B	F	A	D
6	D	B	E	C	F	A
7	A	E	C	D	B	F
8	F	A	D	E	C	B

1) A = vehicle anticholinergic + L-DOPA
2) B = dose 1 anticholinergic + vehicle L-DOPA
3) C = dose 1 anticholinergic + L-DOPA
4) D = dose 2 anticholinergic + vehicle L-DOPA
5) E = dose 2 anticholinergic + L-DOPA
6) F = vehicle anticholinergic + vehicle L-DOPA

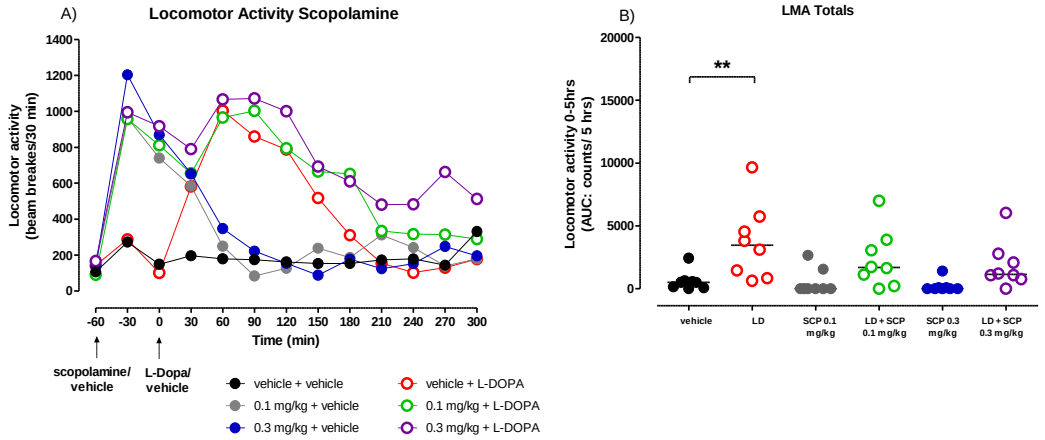


Figure 2.8 Typical example of the effect of scopolamine on L-DOPA-induced locomotor activity in MPTP-treated common marmosets

Scopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n = 8) A) Locomotor activity time course and B) Total locomotor activity (AUC0-5h). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B). A) No statistical analysis performed; (B) Data analysed by repeated measures ANOVA transformed $y=\sqrt{y}$; (B) $F=5.212$; Df (5, 47); $p=0.0011$; followed by Newman-Keuls post hoc test. $^{**}p<0.01$.

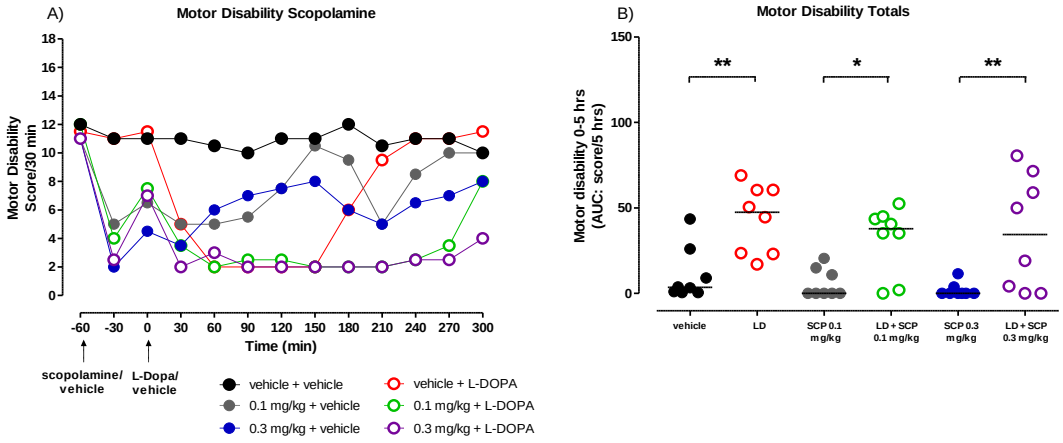


Figure 2.9 Typical example of the effect of scopolamine on L-DOPA-induced reversal of motor disability in MPTP-treated common marmosets

Scopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n = 8) A) Motor disability time course and B) Total motor disability reversal (AUC0-5h). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B). A) No statistical analysis performed; (B) Data analysed by repeated measures ANOVA and transformed $y=\sqrt{y}$; (B) $F=8.247$; Df (5,47); $p<0.0001$ followed by Newman-Keuls post hoc test $^{*}p<0.05$; $^{**}p<0.01$.

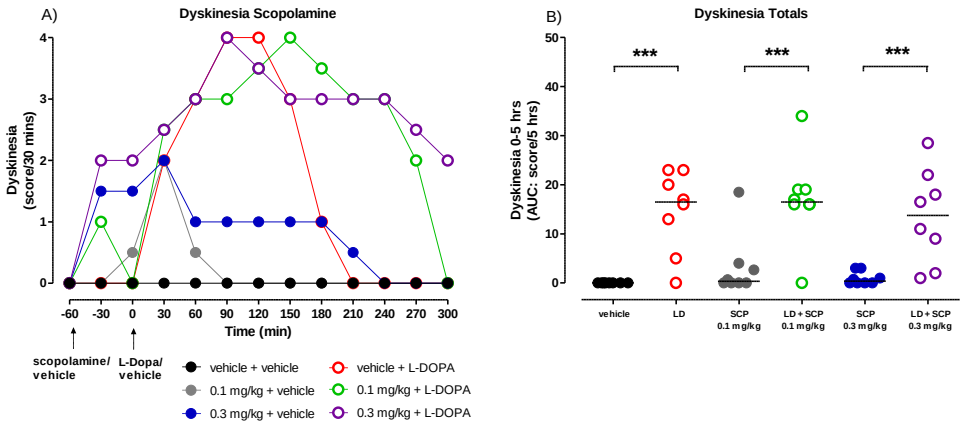


Figure 2.10 Typical example of the effect of scopolamine on L-DOPA-induced dyskinesia in MPTP-treated common marmosets
Scopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n = 8) A) Dyskinesia time course and B) Total dyskinesia ($AUC_{0\text{-}5h}$), Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B). A) No statistical analysis performed; B) Data analysed by repeated measures ANOVA and transformed $y=\sqrt{y}$; $F=14.29$; Df (5, 47); $p<0.0001$followed by Newman-Keuls post hoc test. ***$p<0.001$.

2.2.3.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.02 (San Diego, CA, USA).

Locomotor activity data was collected as 1 minute intervals and then 10 and 30 minute time periods were calculated in Excel for individual animals. The 10 minute period data was used to calculate the locomotor activity "on-time", where locomotor counts greater than 100 per 10 minutes were considered as an increase in activity. The 30 minute data were used to determine the total locomotor activity (AUC).

Motor disability was assessed for 10 minutes every 30 minutes for individual animals. "On-time" for reversal of motor disability was defined as the duration of the test period that an animal scored below 8 on the motor disability scale.

Dyskinesia (dystonia and chorea) was assessed for 10 minutes every 30 minutes for individual animals. "On-time" for dyskinesia (dystonia and chorea) was defined as the duration of the test period that an animal scored above 0 on the dyskinesia rating scale. "On-time > 2" refers to troublesome (marked to severe) dyskinesia (dystonia and chorea) and indicates time when scores are greater than 2. The 30 minute scores were

used to determine the total dyskinesia (dystonia and chorea) (AUC) for individual animals.

Peak activity/score was taken as the maximum count/score achieved per 30 min within the full assessment period.

Area under the curve (AUC) was calculated by GraphPad Prism 5.02 (San Diego, CA, USA) using the trapezoid method. AUC was calculated from the baseline (defined as the 30 min prior administration of anticholinergic) until 6 hr post-treatment for each individual animal.

Time course data were plotted as means at 30 min intervals over 6 hr following drug administration with baseline (pre-treatment) plotted at time -60 (t=-60 min). L-DOPA were administered at t=0 min on the graphs. The statistical analysis was performed as follows:

The statistical analysis for locomotor activity and motor disability data was performed as follows:

1) No statistical analysis was performed on time course data;
2) Totals (AUC_{-1-0h}) and (AUC_{0-5h}) and Peak data were transformed by square root ($Y=\sqrt{Y}$).
3) Totals (AUC_{-1-0h}) and (AUC_{0-5h}), Peak, On-time, On-time > 2 were analysed by Two-way ANOVA (variables: L-DOPA and anticholinergic) and repeated measures ANOVA followed by *post hoc* Newman-Keuls multiple comparisons test.

2.3 General materials

Table 2.3 List of drugs

Item	Supplier
AF-DX 116	HelloBio, UK
Benserazide hydrochloride	Sigma-Aldrich, UK
Benztropine mesylate	Santa Cruz, USA
Darifenacin hydrobromide	Kemprotec Ltd, UK
Levodopa (L-DOPA) methyl ester	Sigma-Aldrich, UK

Methylscopolamine (-scopolamine methyl bromide)	Sigma-Aldrich, UK
NBI-675	Neurocrine Biosciences, Inc, USA
Pilocarpine hydrochloride	Sigma-Aldrich, UK
Pirenzepine dihydrochloride	Sigma-Aldrich, UK
Scopolamine hydrobromide	Sigma-Aldrich, UK
DL-Trihexyphenidyl hydrochloride	Sigma-Aldrich, UK
Tropicamide	Sigma-Aldrich, UK

Table 2.4 List of chemicals and reagents

Item	Supplier
Baytril	Bayer HealthCare A.G, UK
Buprenorphine (Vetergesic)	Reckitt Benckiser Healthcare Ltd, UK
Carprieve 5.0%	Norbrook Laboratories Ltd, UK
Dimethyl sulfoxide (DMSO) sterile	Sigma-Aldrich, UK
EMLA Cream	AstraZeneca, UK
Ethanol	Fisher Scientific, UK
Isoflurane	Abbott Laboratories Ltd, UK
Methyl cellulose	Sigma-Aldrich, UK
Sodium pentobarbitone (Euthanal)	Merial, UK
Sodium pentobarbitone (Dolethal)	Vetoquinol, UK
Sucrose	Merck Biosciences Ltd, UK
Tween 80	Sigma-Aldrich, UK

Table 2.5 List of equipment and consumables

Item	Supplier
23G stainless steel tubing/cannula	Cooper's Needleworks, Birmingham, UK
30G stainless steel tubing/cannula	Cooper's Needleworks, Birmingham, UK
Autoclave	Meadowrose Scientific Ltd, UK
Bulldog clips	World Precision Instruments (WPI), UK
CMA Microinjection Pump	Carnegie Medicin, Sweden

Acrylic denture repair material Dentsply Rapid Repair	Skillbond Direct, High Wycombe, UK
Disposable scalpel no 10	Swann Morton, Sheffield, UK
Hamilton Syringe	ESSLAB, UK
Nanoliter Infusion Pump	WPI, UK
pH meter (pocket)	Camlab Ltd, UK (Shindengen Electric)
Portex fine bore polythene tubing	Sims Portex Ltd, Ashford, UK
Stainless steel screws	Clerkenwell Screws Ltd, London, UK
Stereotaxic Frame	David Kopf Instruments (World Precision Instruments, Stevenage, UK)
Sutures	Ethicon, Johnson & Johnson

Chapter 3 The effect of anticholinergics on pilocarpine-purposeless chewing

3.1. Introduction

Anticholinergics show therapeutic utility in the treatment of some types of dystonia, particularly segmental and generalised forms (Adam & Jankovic, 2007), but tend to induce strong unwanted side effects that limit their use (Cloud & Jinnah, 2010; Lubarr & Bressman, 2011). One of the commonly prescribed relatively M1 selective anticholinergic, trihexyphenidyl, despite its therapeutic effects, has been reported to cause central (drowsiness, confusion, memory problems, hallucinations) and peripheral (urinary retention, constipation, blurry vision and dry mouth) side effects (Goldman & Comella, 2003; Adam & Jankovic; 2007; Cloud & Jinnah, 2010; Lubarr & Bressman, 2011).

The exact mechanism of action of anticholinergics in dystonia is not known, but it is thought that they work by blocking muscarinic ACh receptors in the basal ganglia.
In recent years, considerable effort has been made to better understand and examine the muscarinic receptors activity in motor control. Animal studies show that administration of cholinergic agonists, mainly pilocarpine, induces perioral movements in rats (Salamone *et al.*, 1986; Stewart *et al.*, 1988; Stewart *et al.*, 1989; Mayorga *et al.*, 1999). These involuntary, drug-induced movements are defined in the literature as tremulous, vacuous or purposeless (Salamone *et al.*, 1986; Stewart *et al.*, 1989) and described as rapid vertical deflections of the jaw not directed at any particular object (Cousins *et al.*, 1997; Mayorga *et al.*, 1997). They have been associated with Parkinsonian tremor (Salamone *et al.*, 2001; Betz *et al.*, 2007) and dystonia (Stewart *et al.*, 1988) and are the effect of central stimulation of muscarinic receptor subtypes in the striatum (Salamone *et al.*, 2001). Since pilocarpine is a non-selective cholinergic agonist, it stimulates all subtypes of muscarinic ACh receptors to induce salivation, urination, defecation, lachrymation, piloerection and oral movements (Mayorga *et al.*, 1999).

The central effect of pilocarpine on induction of purposeless chewing is known to be reversed by non-selective and relatively subtype selective anticholinergic agents (Stewart *et al.*, 1989; Mayorga *et al.*, 1999), however, the exact subtype of muscarinic receptor involved in formation of pilocarpine-induced purposeless chewing has been difficult to assess due to the high homology, similarity and distribution of these receptors and poor selectivity of the anticholinergics. All five muscarinic receptors

subtypes are distributed with different intensity in various areas of the brain, including striatum (M1 – M5), hippocampus (M1 – M5), cerebral cortex (M1 – M5), thalamus (M1, M2) and periphery, in the smooth muscle (M2, M3), glands (M1, M3, M5), heart (M2), where they play different functions (Eglen, 2006; Langmead *et al.*, 2008) as described in Chapter 1 section 1.3.2. Muscarinic M1 and M4 receptors are predominantly expressed in the striatum, where they play important role in regulation of motor control. In addition, M1 receptors are in the hippocampus where they have a role in learning and memory. They are also expressed in the periphery, mainly in glands (salivary, gastric, lacrimal) (Eglen, 2006; Langmead *et al.*, 2008) and all these may contribute to reported side effects of anticholinergic treatment (Goldman & Comella, 2003; Adam & Jankovic, 2007; Cloud & Jinnah, 2010; Lubarr & Bressman, 2011). The most recent studies with partially selective M4 antagonist suggested that striatal muscarinic M4 receptors are involved in pilocarpine-induced chewing (Betz *et al.*, 2007) and hence muscarinic M4 receptors may be the target for a development of improved treatment for dystonia with reduced side effects profile.

3.1.1. Hypothesis

It is hypothesised that pilocarpine-induced purposeless chewing, as a model of centrally mediated motor dysfunction generating abnormal involuntary movements, is supressed by anticholinergics through inhibition of muscarinic M4 receptors.

3.1.2. Aims

Specific aims of this study were to:

1) Establish that central muscarinic receptors are responsible for pilocarpine-induced purposeless chewing.
2) Determine the subtypes of muscarinic receptors responsible for pilocarpine-induced purposeless chewing.
3) Determine the role of M4 receptors on pilocarpine-induced purposeless chewing using a novel selective muscarinic M4 antagonist, NBI-675.

3.2 Materials and Methods

In order to address these aims the following studies were performed:

1) The role of central muscarinic receptors in mediation of pilocarpine-induced purposeless chewing in rats was confirmed by treating rats with scopolamine and methylscopolamine 30 min prior to pilocarpine. Purposeless chewing was measured as described below.
2) To confirm subtype of muscarinic receptor involved in mediation of pilocarpine-induced purposeless chewing rats were treated with relatively selective anticholinergics 30 min prior pilocarpine, and the purposeless chewing was assessed as described below.
3) The role of the M4 muscarinic receptor in pilocarpine-induced purposeless chewing was determined using the novel selective M4 antagonist NBI-675. NBI-675 was administered 60 min prior to pilocarpine, and purposeless chewing was measured as described below.

Detailed methodology is described below.

3.2.1 Animal husbandry

Male Wistar rats (160 – 300 g, Harlan, UK) were housed in groups of 4 – 5 per cage at a temperature of 22 ± 2ºC and humidity controlled housing unit on a 12 hours light – dark cycle. Water and food were available *ad libitum* except during the surgery and experiments. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 under UK Home Office Project licence number 70/6898 and 70/7977, approved by the King's College London Ethical Review Panel.

3.2.2 Assessment of pilocarpine-induced purposeless chewing

Systemic administration of pilocarpine to rats induces purposeless chewing movements, as described in Chapter 2, section 2.2.1.2. Animals were placed in a Perspex observation boxes (21 x 35 x 17 cm) with mirrors placed behind to allow for the viewing of animal from several angles and had 30 min of acclimatisation to the testing room before the experiment. Pilocarpine (3.4 mg/kg i.p.) was administered 30 min after anticholinergics administration. Purposeless chewing was assessed before and every 10 min after pilocarpine administration and the number of movements was

recorded for 1 min every 10 min using a mechanical hand counter as described in section 2.2.1.2.

3.2.3 Drug treatment

3.2.3.1 Peripheral administration of anticholinergics

DL-Trihexyphenidyl hydrochloride (0.3 – 5 mg/kg), benztropine mesylate (0.3 – 5 mg/kg), darifenacin hydrobromide (0.75 – 24 mg/kg), tropicamide (1.25 – 20 mg/kg), AF-DX 116 (1 – 30 mg/kg), scopolamine hydrobromide (0.01 – 1 mg/kg), (-) scopolamine methyl bromide (methylscopolamine) (0.01 – 1 mg/kg) and NBI-675 (0.1 – 30 mg/kg) were administered i.p. 30 min prior pilocarpine (3.4 mg/kg i.p.). Doses of the drugs were chosen according to previously published studies (Stewart *et al.*, 1989; Mayorga *et al.*, 1999; Betz *et al.*, 2007) or according to the PK and PD studies (NBI-675) received from Neurocrine Biosciences, Inc.

Trihexyphenidyl was dissolved in deionised water; darifenacin and AF-DX 116 were dissolved in a sterile dimethyl sulphoxide (DMSO) and diluted in 0.9% saline (for darifenacin 5:1 saline to DMSO, and for AF-DX 116 3:1 saline to DMSO) and tropicamide was dissolved in a minimal amount of ethanol (200 µl) and diluted in 0.9% saline. NBI-675 was dissolved in 5% Tween80 and 0.5% methylcellulose made up in deionised water, vortexed and sonicated for 1 hr. The pH was adjusted to 4 – 6 with 1M NaOH. All other drugs were dissolved in 0.9% saline and administered intraperitoneally at a dose volume of 1 ml/kg, except the highest dose of AF-DX 116 (30 mg/kg) which, due to the problems with solubility, was administered at a dose volume of 2 ml/kg.

Drug treatments were administered on a crossover design, so each rat was treated with all doses of a single drug or vehicle with at least 48 hr between the tests. Typical latin square used to randomise drug treatments in shown in Table 3.1. Different animal groups were used to assess the effect of anticholinergics. Benztropine, trihexyphenidyl, tropicamide and darifenacin were assessed in one group of rats (group I); NBI-675, scopolamine and methylscopolamine – group II; pirenzepine and AF-DX-116 – group III. Drugs were tested individually in separate studies, following by at least a week of wash-out between the drugs.

Table 3.1 Typical latin square with a crossover designed treatment for administration of different doses of anticholinergics prior to pilocarpine.

Animal number	Treatment day					
	1	2	3	4	5	6
1	F	D	C	B	E	A
2	B	A	F	D	C	E
3	D	E	A	C	B	F
4	F	E	B	A	D	C
5	E	C	D	F	A	B
6	F	B	C	E	A	D
7	A	D	E	C	B	F
8	E	F	C	D	B	A

A = vehicle + pilocarpine
B = dose 1 + pilocarpine
C = dose 2 + pilocarpine
D = dose 3 + pilocarpine
E = dose 4 + pilocarpine
F = dose 5 + pilocarpine

3.2.3.2 Central administration of anticholinergics

3.2.3.2.1 Stereotaxic guide cannula implantation into the lateral ventricle

In order to administer drugs centrally, a stainless steel guide cannula (23 G, Coopers Needle Works Ltd, UK) was implanted into the lateral ventricle (AP: -0.8 mm; ML: -1.4 mm; DV: -2.8 mm) (Fig. 3.1) using standard stereotaxic techniques. Rats were placed in an induction chamber and anesthetised with isoflurane 4% – 5% in 100% medical oxygen (BOC, Manchester, UK). Animals were then transferred into the Kopf stereotaxic frame (Fig. 3.2) with the incisor bar set at -3.3 mm and ear bars positioned symmetrically. Isoflurane anaesthesia (2.5% – 3% in medical oxygen) was maintained throughout through a mask. The body temperature was maintained at 37°C using thermostatic heating pad and a rectal probe. After shaving and disinfecting the head, a topical local anaesthetic cream (EMLA cream, Astra Zeneca) was applied. An anterior to posterior skin incision was made and the skull exposed. Connective tissue was cleared using a disposable blade, and bulldog clips were used to retract the skin. Bregma was located and coordinates recorded. A hole was drilled through the skull directly above the point of insertion and a stainless steel guide 23 G cannula was lowered into the lateral ventricle. Coordinates for location of the guide cannula relative to bregma are shown in Figure 3.1 (Paxinos & Watson, 1986). The cannula was held in place using acrylic denture cement (Rapid Repair, Skillbond Direct, UK) attached to the skull by two stainless steel screws (Clerkenwell Screws Ltd, UK). A stainless steel stylet was inserted into the cannula to keep it free from blockage. The wound was

closed using two to three separate stitches of dissolvable vicryl sutures. EMLA cream was applied on the sutured wound. Animals were given 5 ml of 0.9 % saline subcutaneously (s.c.) to prevent dehydration and the analgesic buprenorphine (0.2 mg/kg s.c.) and analgesic/anti-inflammatory carprieve (5 mg/kg s.c.) were administered. Animals were placed in cages with a heat-controlled pad underneath until completely recovered from anaesthesia. The post-operative weight was monitored daily (Fig. 3.3) and a mash diet (softened food pellets) provided until animals regained their pre-operative weight. They were allowed 6 days to recover prior behavioural testing.

The placement of the cannula was verified by microinjection of a trypan blue dye (1 µl) into the lateral ventricle, in one animal from each group (every 7^{th} – 8^{th} animal).

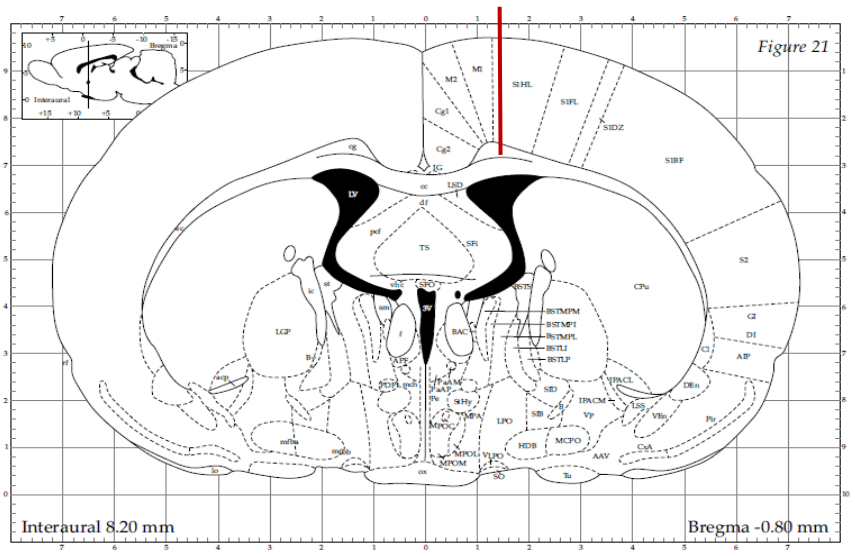


Figure 3.1 Location of cannula implanted in the right lateral ventricle (red line). Modified from Paxinos & Watson (1986).

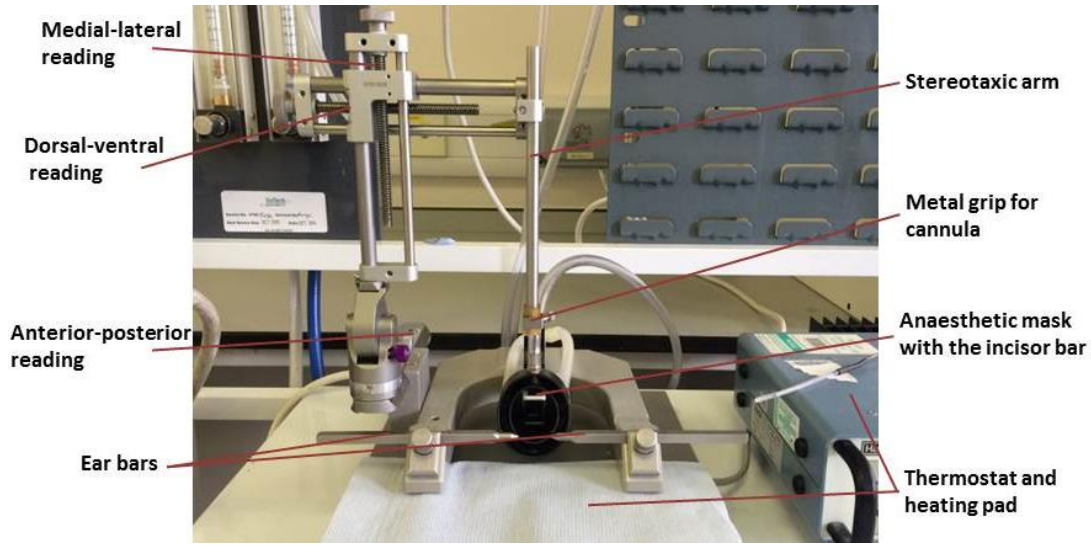


Figure 3.2 Stereotaxic frame for the cannula implantation.

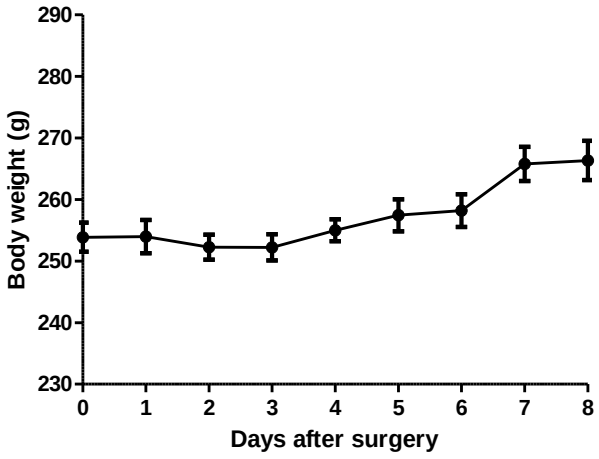


Figure 3.3 Typical example of body weight changes post cannula implantation into the lateral ventricle.
Day 0 is a day of the surgery. Data are expressed as mean ± SEM (n = 8) of body weight of animals.

3.2.3.2.2 Intracerebroventricular administration of anticholinergics

Pirenzepine dihydrochloride (2.4 – 377 nmol/µl i.c.v., equivalent of 1 – 160 µg/µl) was dissolved in 0.9% saline and, due to limited solubility, administered in a dose volume of 2 µl and 3 µl (the highest dose) at a rate 1 µl/min. Darifenacin hydrobromide (0.2 – 295.5 nmol/µl, equivalent of 0.1 – 150 µg/µl) was dissolved in a sterile DMSO and diluted with 0.9% saline (4:1 saline to DMSO) and administered i.c.v. at a dose volume of 2 µl at a rate 1 µl/min. Trihexyphenidyl (3 – 59 nmol, equivalent of 1 – 20

µg/µl) was dissolved in a deionised water and administered i.c.v. at a dose volume of 1 µl (3 nmol), 2 µl (29.5 nmol) and 4 µl (59 nmol), due to poor solubility, at a rate 1 µl/min. Tropicamide (3.5 – 422 nmol/ µl, equivalent of 1 – 120 µg/µl) was dissolved in a minimal amount of ethanol (20 µl) and diluted in 0.9% saline and administered i.c.v. at a dose volume of 2 µl at a rate 1 µl/min.

A stainless steel guide cannula was stereotaxically implanted into the lateral ventricle of rats, as described in section 3.2.3.2.1, in order to allow repeated microinjection of anticholinergics or vehicle.

Drug injection was made through a stainless steel injection needle (30 G, Coopers Needle Works Ltd, UK) placed inside the guide cannula. Injection needles were 1 mm longer than the tip of guide cannula (coordinates of injection site: AP: -0.8 mm; ML: -1.4 mm; DV: -3.8 mm). Animals were hand restrained (Fig. 3.4). The end of injection needle was connected via tubing to the 100 µl Hamilton gastight syringe containing anticholinergic or vehicle (saline) and attached to the CMA Microinjection Pump (Carnegie Medicin, Sweden) or Nanoliter Infusion Pump (WPI, UK) (Fig. 3.4). Total injected volume was either 1 – 4 µl (as stated above) at a rate of 1 µl/min. Anticholinergics administered centrally (i.c.v.) were assessed on individual groups of rats (one anticholinergic per group).

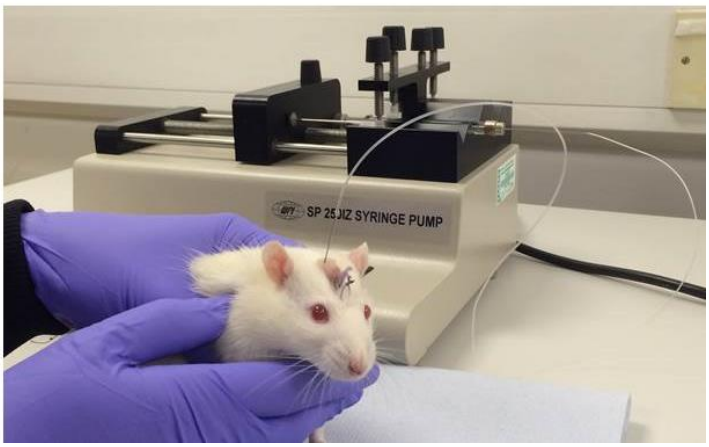


Figure 3.4 Microinjection of substances via guide cannula implanted to the brain.
Rat is being hand restrained and the end of injection needle is connected via tubing to a 100 µl Hamilton syringe with a drug solution and attached to a Nanoliter Infusion Pump.

Injection needle was left in place for a further 4 min to allow drug diffusion into the surrounding area and to prevent reflux up the needle tract and cannula. Injections were conducted in a randomised manner according to a modified Latin square design, at least 48 hrs between the tests (Table 3.1). Drugs were tested individually in separate studies. Rats were placed in boxes and assessment of chewing was conducted as described in section 2.2.3.1.

3.2.4 Statistical analysis

Data and statistical analysis for pilocarpine-induced purposeless chewing were performed using GraphPad Prism 5.02 (San Diego, CA, USA). Area under curve (AUC) for the time course was calculated by trapezoid method. Data are expressed as mean ± SEM and were analysed by sigmoidal nonlinear regression analysis 3-parameter fit. ID_{50} value was derived from the curve fit. Bottom was constrained to the value 0. Differences between treatment and vehicle control for AUC data was analysed by non-parametric Friedman's test followed by a *post hoc* Dunn's test.

Peripheral effects of anticholinergics on pilocarpine-induced salivation, piloerection, defecation, urination, lachrymation were measured qualitatively and scored for a degree of inhibition (+ low inhibition; ++ moderate inhibition; +++ high inhibition).

3.3. Results

Based on results from Chapter 2, section 2.2.1.4, an ED_{50} dose of pilocarpine (3.4 mg/kg) was chosen to induce purposeless chewing in rats in these studies. As expected, this dose of pilocarpine induced chewing behaviour which was visible within few minutes of administration, peaked between 10 – 20 min, and lasted about 100 min. Baseline number of pilocarpine-induced chews differs between animals as the experiments were conducted on different groups of animals.

Overall, pre-treatment with anticholinergics resulted in a dose-dependent inhibition of pilocarpine-induced purposeless chewing, which was consistent for all centrally acting agents tested. Administration of vehicle did not alter chewing behaviour in rats.

3.3.1 Central effect of pilocarpine-induced chewing

In order to confirm that pilocarpine-induced chewing is mediated through central muscarinic receptor, the effect of the centrally acting anticholinergic scopolamine, and its peripherally acting derivative, methylscopolamine, were investigated.

Scopolamine (0.01 – 1 mg/kg), a non-selective centrally acting antimuscarinic, produced dose-related inhibition of purposeless chewing induced by pilocarpine (Fig. 3.5 A). Scopolamine significantly reduced purposeless chewing at the two higher doses (0.1 and 1 mg/kg) (Fig. 3.5 B). There was a nearly complete inhibition of purposeless chewing at the highest dose administered (1 mg/kg) (Fig. 3.5 A). The ID_{50} for scopolamine was 0.04 mg/kg (95% CI = 0.02 – 0.06 mg/kg).

Peripherally acting methylated derivative of scopolamine, methylscopolamine (0.01 – 1 mg/kg), produced total suppression of pilocarpine-induced purposeless chewing accounting for 16% when compared to vehicle-treated animals, which was significant for 0.01 and 1 mg/kg (Fig. 3.6 A & B).

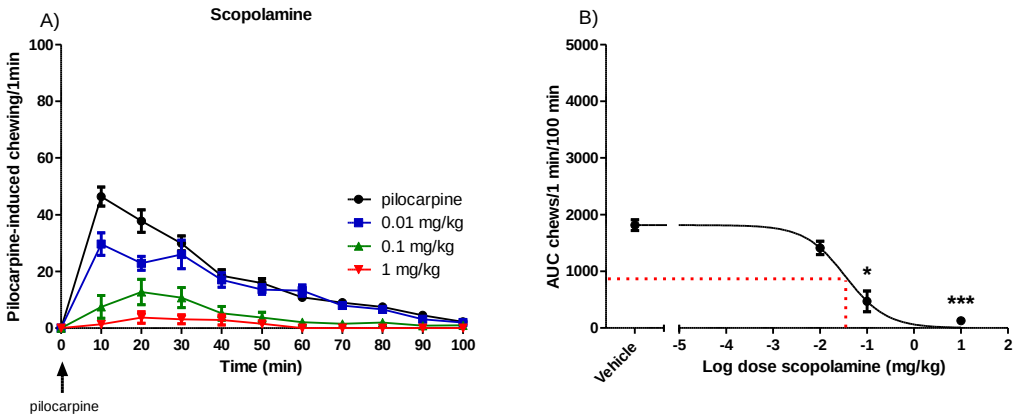


Figure 3.5 Effect of scopolamine (i.p.) on pilocarpine-induced purposeless chewing.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for scopolamine (0.01 – 1 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} = 0.04 ± 1.4 mg/kg (dotted line); (95% CI = 0.02 – 0.07 mg/kg; r^2 = 0.81 p< 0.05); *p<0.05; ***p<0.001 compared to vehicle-treated animals (Friedman's test (FS = 18.75; ***p<0.001; p=0.0003) followed by post hoc Dunn's test).

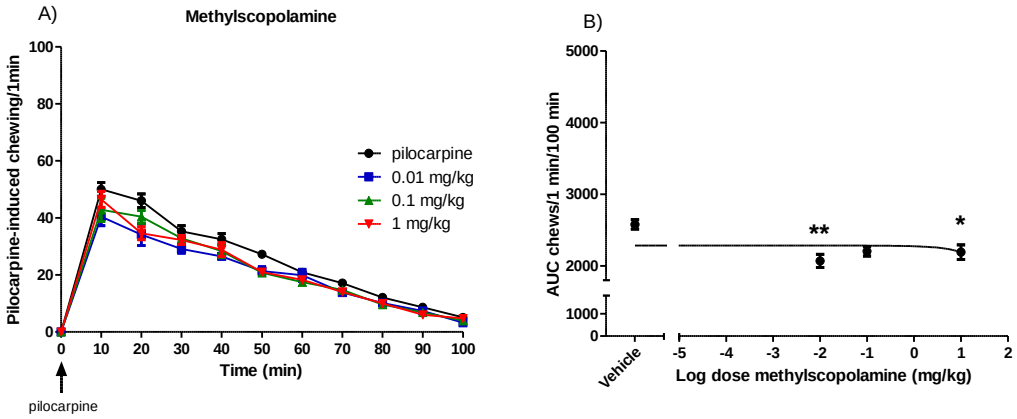


Figure 3.6 Effect of methylscopolamine (i.p.) on pilocarpine-induced purposeless chewing.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for methylscopolamine (0.01 – 1 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} > 1 mg/kg; r^2 = 0.02 NS; *p<0.05; **p<0.01 compared to vehicle-treated animals (Friedman's test (FS = 10.95; p<0.05; p=0.0120) followed by post hoc Dunn's test).

3.3.2 The effect of subtype selective anticholinergics

3.3.2.1 Muscarinic M1 selective anticholinergics

Peripheral administration of trihexyphenidyl (0.3 – 5 mg/kg i.p.), an M1 selective muscarinic antagonist, 30 min before pilocarpine, produced a dose-dependent inhibition of pilocarpine-induced purposeless chewing (Fig. 3.7 A). Trihexyphenidyl significantly reduced chewing at doses of 1.25 – 5 mg/kg when compared to vehicle-treated animals (Fig. 3.7 B). The ID_{50} for trihexyphenidyl was 1.6 mg/kg (95% CI = 1.2 – 2.2 mg/kg).

Central administration of trihexyphenidyl (3 – 59 nmol i.c.v.) into the lateral ventricle resulted in a small inhibition of pilocarpine-induced purposeless chewing (Fig. 3.8 A & B). Purposeless chewing was significantly reduced at the doses of 3 and 29.5 nmol when compared to vehicle-treated animals. However, this inhibition amounted to about 36% at all doses tested. Higher doses could not be tested due to the poor solubility of the compound.

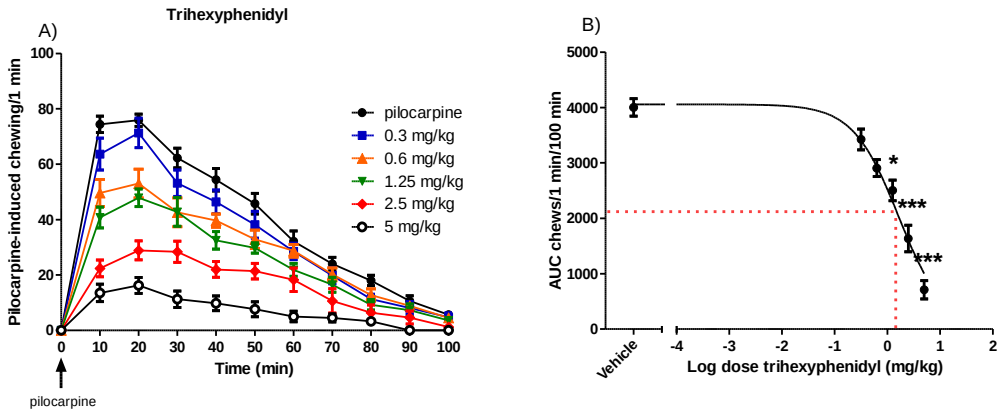


Figure 3.7 Effect of trihexyphenidyl (i.p.) on pilocarpine-induced purposeless chewing. A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for trihexyphenidyl (0.3 – 5 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} = 1.6 ± 1.1 mg/kg (dotted line); (95% CI = 1.2 – 2.2 mg/kg; r^2 = 0.82 $p < 0.05$); *$p<0.05$; ***$p<0.001$ compared to vehicle-treated animals (Friedman's test (FS = 35.71; ***$p<0.001$; $p<0.0001$) followed by post hoc Dunn's test).

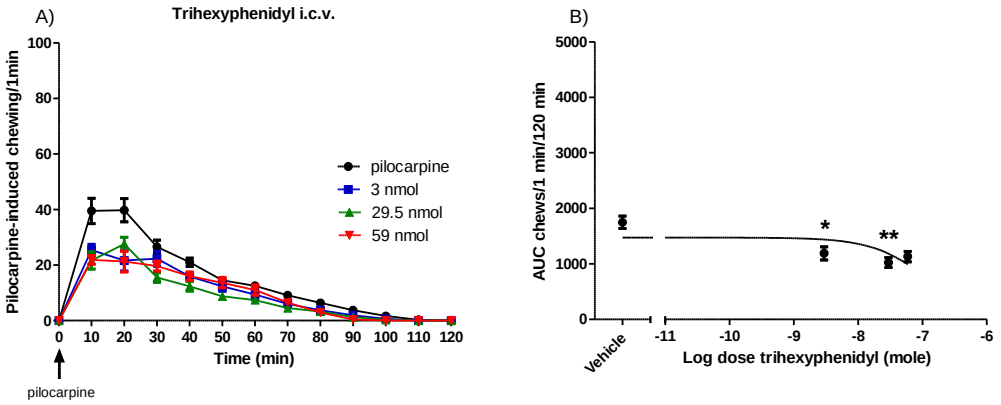


Figure 3.8 Effect of trihexyphenidyl (i.c.v.) on pilocarpine-induced purposeless chewing. A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for trihexyphenidyl (3 – 59 nmol i.c.v., equivalent to 1 – 20 µg) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} > 59 nmol; (r^2 = 0.21 NS); *p<0.05; **p<0.01 compared to vehicle-treated animals (Friedman's test (FS = 10.95; *p<0.05; p=0.0120) followed by post hoc Dunn's test).

Benztropine (0.3 – 5 mg/kg i.p.), a selective M1 anticholinergic, produced a dose-dependent reduction of pilocarpine-induced purposeless chewing behaviour (Fig. 3.9 A). Benztropine significantly reduced pilocarpine-induced purposeless chewing except the lowest dose (0.3 mg/kg). The ID_{50} for benztropine was 0.7 mg/kg (95% CI = 0.5 – 0.9 mg/kg). At the highest dose tested little or no chewing was observed (Fig. 3.9 A).

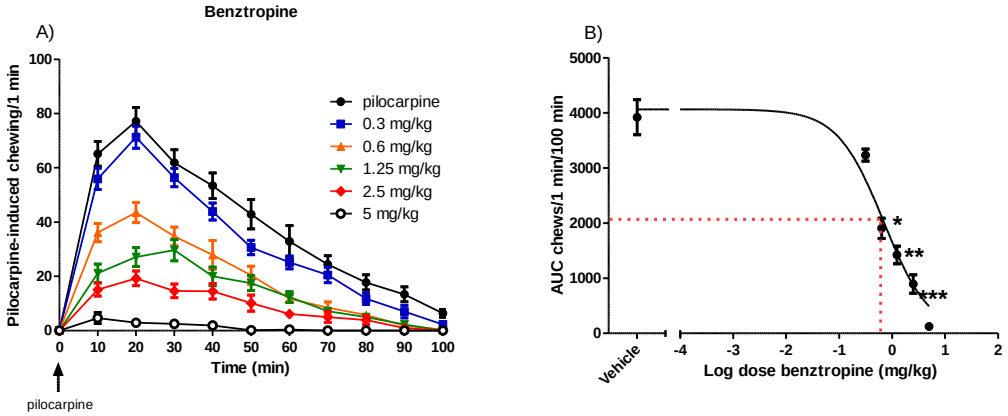


Figure 3.9 Effect of benztropine (i.p.) on pilocarpine-induced purposeless chewing.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for benztropine (0.3 – 5 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} = 0.7 ± 1.1 mg/kg (dotted line); (95% CI = 0.5 – 0.9 mg/kg; r^2 = 0.84 $p<0.05$); *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to vehicle-treated animals (Friedman's test (FS = 36.07; ***$p<0.001$; $p<0.0001$) followed by post hoc Dunn's test).

Systemic administration of putative M1 selective antagonist, pirenzepine (0.1 – 50 mg/kg i.p.) produced total suppression of pilocarpine-induced chewing accounting for 18% when compared to vehicle-treated animals, which was significant for 1 and 10 mg/kg (Fig. 3.10 A & B).

Central administration of putative M1 selective antagonist, pirenzepine (2.4 – 377 nmol i.c.v.) into the lateral ventricle resulted in a dose-related inhibition of purposeless chewing induced by pilocarpine (Fig. 3.11 A), which was significant for the two highest doses (189 and 377 nmol) (Fig. 3.11 B), with ED_{50} of 29 nmol (95% CI = 19 – 43.5 nmol). The highest dose of pirenzepine (377 nmol) completely blocked purposeless chewing.

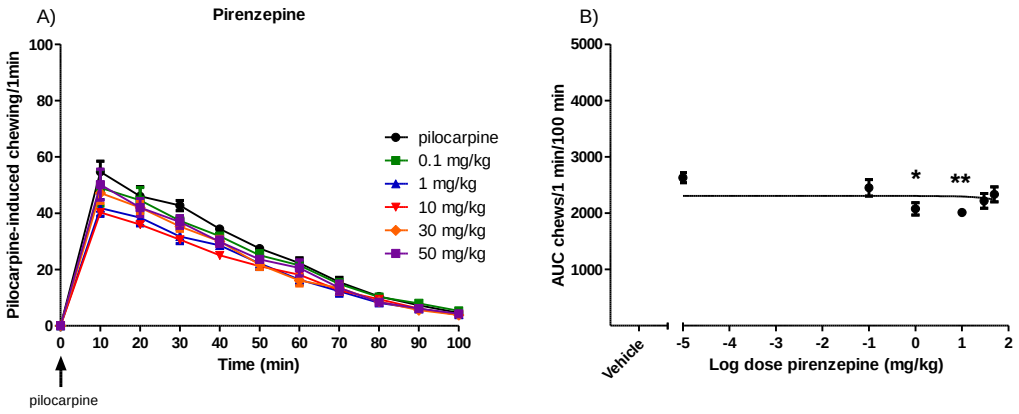


Figure 3.10 Effect of pirenzepine (i.p.) on pilocarpine-induced purposeless chewing.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for pirenzepine (0.1 – 50 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} > 50 mg/kg; r^2 = 0.005 NS; *p<0.05; **p<0.01 compared to vehicle-treated animals (Friedman's test (p<0.01; FS = 15.08; p=0.010) followed by post hoc Dunn's test).

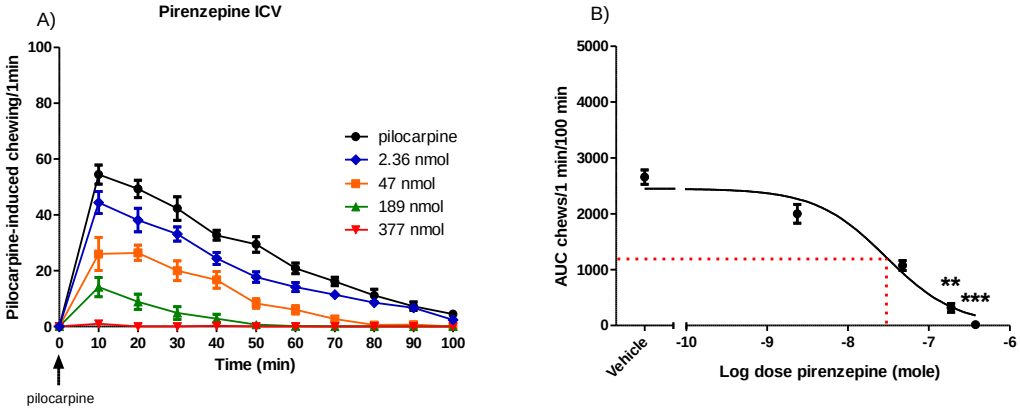


Figure 3.11 Effect of pirenzepine (i.c.v.) on pilocarpine-induced purposeless chewing.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for pirenzepine (2.4 – 377 nmol i.c.v., equivalent to 1 – 160 µg) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} = 29 ± 1.2 nmol (dotted line); (95% CI = 19.0 – 45.5 nmol; r^2 = 0.90 p<0.05); **p<0.01; ***p<0.001 compared to vehicle-treated animals (Friedman's test (FS = 28.00; ***p<0.001; p<0.0001) followed by post hoc Dunn's test).

3.3.2.2 Muscarinic M2 selective anticholinergics

AF-DX 116 (1 – 30 mg/kg i.p.), an M2 selective antagonist, significantly inhibited pilocarpine-induced purposeless chewing only at the top dose (30 mg/kg) (Fig. 3.12 A & B), however, higher doses could not be tested due to the poor solubility of the drug, so the ID_{50} could not be determined.

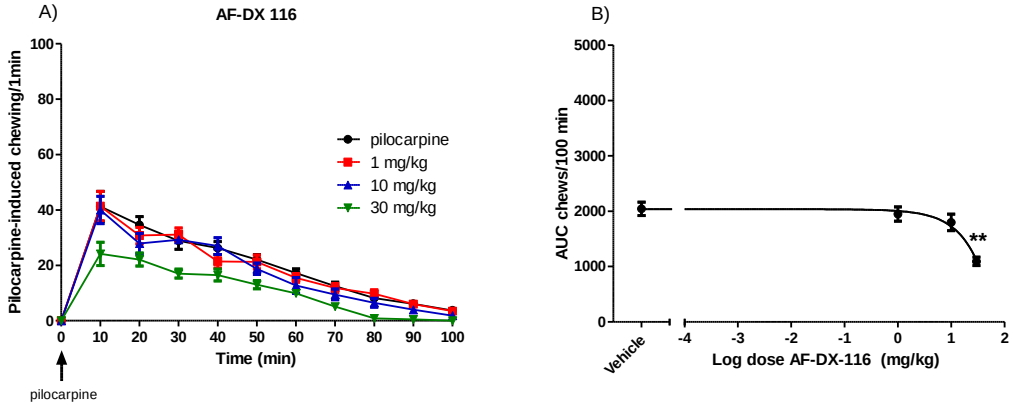


Figure 3.12 Effect of AF-DX 116 (i.p.) on pilocarpine-induced purposeless chewing.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for AF-DX 116 (1 – 30 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} > 30 mg/kg; (r^2 = 0.56 NS) **p<0.01 compared to vehicle-treated animals (Friedman's test (FS = 15.45; **p<0.01; p=0.0015) followed by post hoc Dunn's test).

3.3.2.3 Muscarinic M3 selective anticholinergics

Systemic administration of darifenacin (0.75 – 24 mg/kg), an M3 selective antagonist, produced dose-dependent inhibition of pilocarpine-induced chewing (Fig. 3.13 A). Darifenacin significantly reduced chewing at three higher doses (6 – 24 mg/kg) (Fig. 3.13 B) when compared to vehicle-treated animals. The ID_{50} for darifenacin was 9 mg/kg (95% CI = 7.2 – 11.3 mg/kg).

Central administration of darifenacin (0.2 – 295.5 nmol i.c.v.) into the lateral ventricle resulted in a small and variable inhibition of pilocarpine-induced chewing (Fig. 3.14 A). Chewing was significantly reduced following doses of 2 – 295.5 nmol when compared to vehicle-treated animals. The complete inhibition of chewing was not

achieved and the highest dose tested (295.5 nmol) produced only moderate suppression of chewing (Fig. 3.14 B).

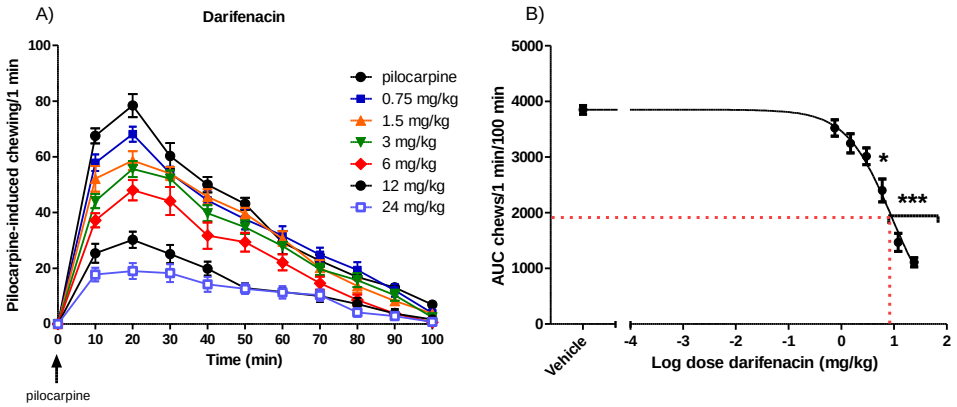


Figure 3.13 Effect of darifenacin (i.p.) on pilocarpine-induced purposeless chewing. A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for darifenacin (0.75 – 24 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} = 9 ± 1.1 mg/kg (dotted line); (95% CI = 7.2 – 11.3 mg/kg; r^2 = 0.85 $p<0.05$); *$p<0.05$; ***$p<0.001$ compared to vehicle-treated animals (Friedman's test (FS = 40.87; ***$p<0.001$; $p<0.0001$) followed by post hoc Dunn's test).

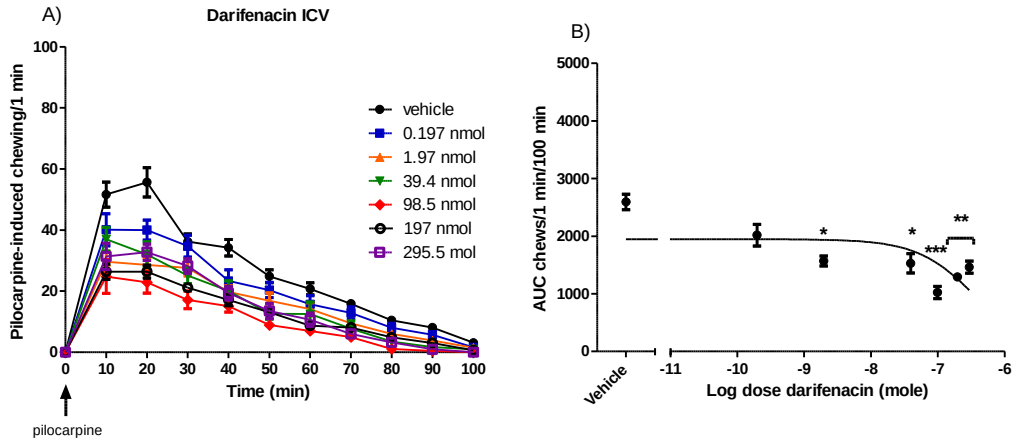


Figure 3.14 Effect of darifenacin (i.c.v.) on pilocarpine-induced purposeless chewing. A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for darifenacin (0.2 – 295.5 nmol i.c.v., equivalent to 0.1 – 150 µg) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} > 295.5 nmol; (r^2 = 0.24 NS); *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to vehicle-treated animals (Friedman's test (FS = 32.25; ***$p<0.001$; $p<0.0001$) followed by post hoc Dunn's test).

3.3.2.4 Muscarinic M4 selective anticholinergics

Tropicamide (1.25 – 20 mg/kg), the putative M4 selective anticholinergic, dose-dependently suppressed pilocarpine-induced chewing in rats (Fig. 3.15 A) when administered peripherally. Chewing was significantly reduced at the three higher doses (5 – 20 mg/kg) (Fig. 3.15 B) with ID_{50} of 8.6 mg/kg (95% CI = 6.3 – 11.8 mg/kg). Nevertheless, complete inhibition of chewing was not observed with the highest dose tested.

Central administration of tropicamide (3.52 – 422 nmol i.c.v.) into the lateral ventricle showed no effect on pilocarpine-induced purposeless chewing in rats (Fig. 3.16 A & B). Higher doses were not tested due to the poor solubility of the compound.

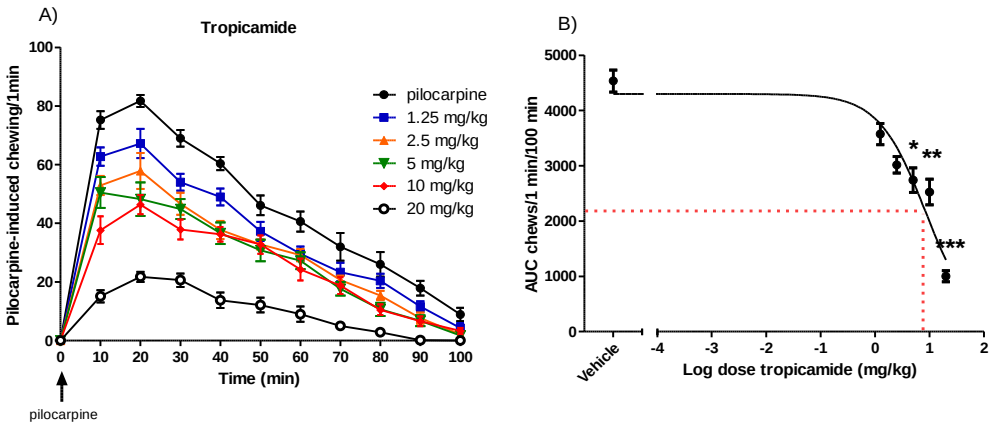


Figure 3.15 Effect of tropicamide (i.p.) on pilocarpine-induced purposeless chewing. A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for tropicamide (1.25 – 20 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} = 8.6 ± 1.2 mg/kg (dotted line); (95% CI = 6.3 – 11.8 mg/kg; r^2 = 0.76 $p<0.05$); *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to vehicle-treated animals (Friedman's test (FS = 31.77; ***$p<0.001$; $p<0.0001$) followed by post hoc Dunn's test).

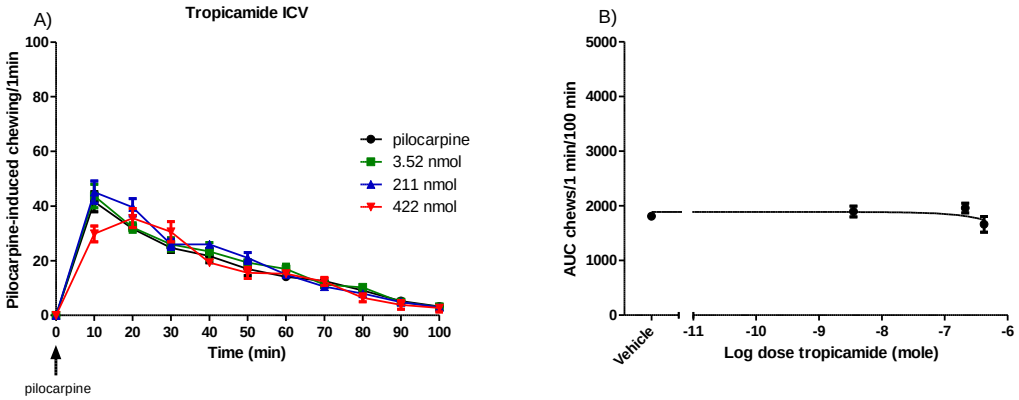


Figure 3.16 Effect of tropicamide (i.c.v.) on pilocarpine-induced purposeless chewing. A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for tropicamide (3.52 – 422 nmol i.c.v., equivalent to 1 – 120 µg) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} > 422 nmol; r^2 = 0.06 NS; NS compared to vehicle-treated animals (Friedman's test (FS = 7.971; NS; p=0.0466)).

The novel M4 selective antagonist NBI-675 (0.1 – 30 mg/kg i.p.) produced a dose-related inhibition of pilocarpine-induced purposeless chewing (Fig. 3.17 A). NBI-675 significantly reduced chewing at the two highest doses (10 and 30 mg/kg) (Fig. 3.17 B). The highest dose (30 mg/kg) of NBI-675 resulted in a nearly complete inhibition of pilocarpine-induced chewing. The ID_{50} for NBI-675 was 9.5 mg/kg (95% CI = 6.7 – 13.5 mg/kg).

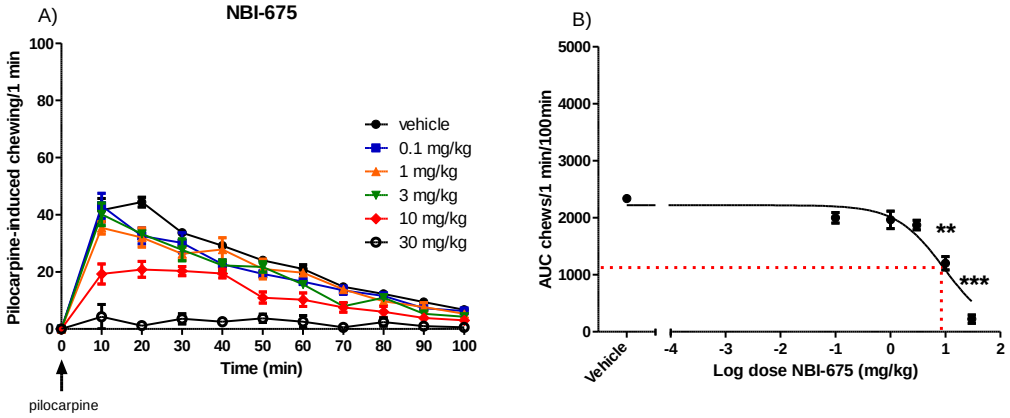


Figure 3.17 Effect of NBI-675 (i.p.) on pilocarpine-induced purposeless chewing.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for NBI-675 (0.1 – 30 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) purposeless chewing (AUC time course). Data were analysed by a non-linear curve fit. Bottom was constrained to 0. ID_{50} = 9.5 ± 1.2 mg/kg (dotted line); (95% CI = 6.7 – 13.5 mg/kg; r^2 = 0.83 $p<0.05$); **$p<0.01$; ***$p<0.001$ compared to vehicle-treated animals (Friedman's test (FS = 29.69; ***$p<0.001$; $p<0.0001$) followed by post hoc Dunn's test).

Table 3.2 Summary of the inhibitory effect of anticholinergics on pilocarpine-induced chewing.
- no effect; + small inhibition; ++ moderate inhibition; +++ high inhibition.

Anticholinergics	Subtype	Effect
Scopolamine i.p.	Non-selective	+++
Methylscopolamine i.p.	Non-selective	-
Trihexyphenidyl i.p.	M1	+++
Trihexyphenidyl i.c.v.	M1	+
Benztropine i.p.	M1	+++
Pirenzepine i.p.	M1	-
Pirenzepine i.c.v.	M1	+++
AF-DX 116 i.p.	M2	++
Darifenacin i.p.	M3	++
Darifenacin i.c.v.	M3	+
Tropicamide i.p.	M4	+++
Tropicamide i.c.v.	M4	-
NBI-675 i.p.	M4	+++

3.3.3 Other effects of anticholinergic treatment

Pilocarpine produced peripheral side effects including increased salivation, diarrhoea, urination, lachrymation, piloerection, which were visible within few minutes of administration. Within few minutes of administration all anticholinergics suppressed

these peripheral effects induced by pilocarpine (Table 3.3), which was observed during the assessment of chewing, but not quantified.

In addition, higher doses of centrally acting anticholinergics not only reduced pilocarpine-induced chewing movements and peripheral effects, but also some compounds, specifically pirenzepine and NBI-675, produced CNS effects which were observed in animals. These were manifested by reduced activity and movement, recumbency, and overall somnolence, which could be an indication of the CNS anticholinergics effects.

Table 3.3 Effect of inhibition of pilocarpine-induced peripheral side effects of different anticholinergics following intraperitoneal administration measured qualitatively during assessment of chewing.

The effects are based on the middle dose.

+ low inhibition; ++ moderate inhibition; +++ high inhibition; NS non-selective

Anticholinergic	Selectivity	Salivation	Piloerection	Defecation	Urination	Lachrymation
Scopolamine	NS	+++	+++	+++	+++	++
Methylscopolamine	NS	+++	+++	+++	+++	+++
Trihexyphenidyl	M1	++	+++	+++	+++	++
Benztropine	M1	++	+++	+++	+++	++
Pirenzepine	M1	+++	+++	+++	+++	+++
AF-DX 116	M2	++	+++	+++	+++	+
Darifenacin	M3	+++	++	+++	+++	+++
Tropicamide	M4	++	++	++	++	++
NBI-675	M4	+	++	++	++	++

3.4. Discussion

The studies described in this chapter focused on examining the effect of muscarinic antagonists on suppression of pilocarpine-induced purposeless chewing in rats. Since striatally located muscarinic acetylcholine (mACh) M4 receptors are thought to be responsible for the production of purposeless chewing movements in rats (Salamone *et al.*, 1986; Stewart *et al.*, 1989; Salamone *et al.*, 1990) it was hypothesised that centrally acting M4 antagonists could be utilized clinically to aid regulation of abnormal movements associated with motor dysfunction, such as dystonia.

This hypothesis was tested using anticholinergics with different relative selectivity to muscarinic receptors subtype, including the novel selective M4 antagonist, NBI-675, on inhibition of pilocarpine-induced purposeless chewing in rats.

3.4.1 The effect of peripherally administered anticholinergics

Administration of a non-selective centrally acting scopolamine nearly completely inhibited purposeless chewing, and this agrees with previous findings (Salamone *et al.,* 1986; Stewart *et al.,* 1989). Moreover, its peripherally acting methylated derivative, methylscopolamine, showed no effect on pilocarpine-induced purposeless chewing. These findings are in agreement with observation by Stewart *et al.,* (1989) who demonstrated inhibitory effect of methylscopolamine on pilocarpine-induced purposeless chewing only when administered via central route into the lateral ventricle, and not via systemic route (Stewart *et al.,* 1988). In addition to the above, systemic administration of pirenzepine, an M1 selective antagonist, was unable to block pilocarpine-induced chewing. This result is not unexpected, knowing its poor brain penetrant properties (Carmine & Brogden, 1985). It has, however, blocked peripheral effects of pilocarpine, including piloerection, salivation, defecation, urination and lachrymation. Therefore, these results confirm first aim of the study that centrally located muscarinic receptor is responsible for mediation of purposeless chewing.

Overall, some drugs were more potent that others in suppression of pilocarpine-induced purposeless chewing in the current study, and this could correspond to the relative potency of the compounds used (Chapter 1 section 1.3.2.1). The rank order of potency, according to the obtained ID_{50} values for i.p. administration, on inhibition of purposeless chewing was scopolamine > benztropine > trihexyphenidyl > NBI-675 =

tropicamide > darifenacin > AF-DX 116 > pirenzepine = methylscopolamine. However, these values are dependent on PK and brain penetration, and not subtype selectivity.

The relative inhibition of purposeless chewing, binding affinities of these drugs to muscarinic receptors subtypes (Chapter 1, section 1.3.2.1) and central location of muscarinic receptor subtypes, suggest that muscarinic M2 and M3 receptors have a minor role in mediation of purposeless chewing (Waelbroeck *et al.*, 1990; Eglen, 2006; Langmead *et al.*, 2008). AF-DX 116 (M2) and darifenacin (M3) showed the least potency in inhibition of purposeless chewing movements. Although, evidence suggests that these receptors are found in the striatum, they are not highly expressed in this area of the brain (Waelbroeck *et al.*, 1990). AF-DX 116 evoked partial inhibitory effect on pilocarpine-induced chewing. These data confirm results from study by Stewart and colleagues (1989), where AF-DX 116 showed moderate inhibition of pilocarpine-induced chewing, for both central and systemic administration of the compound. According to their study, a maximum inhibition of chewing was seen at the dose of 30 mg/kg and above, for systemic administration, and the effect of increasing doses of AF-DX 116 was not significant. Due to a poor solubility of the compound, maximal dose used in the current study was 30 mg/kg, and it cannot be fully concluded whether the maximal inhibition of chewing was seen at that dose.

Likewise, peripheral injection of darifenacin showed a moderate effect on inhibition of pilocarpine-induced purposeless chewing. Darifenacin is an M3 selective compound and muscarinic M3 receptors are expressed in low levels in the CNS (Eglen, 2006; Langmead *et al.*, 2008). As stated in Chapter 1, high doses and high homology of different subtypes of muscarinic receptors to each other may lead to the possibility that the compound has lost its selectivity or might be acting on other subtypes of muscarinic receptors, or even other types of receptors in the brain. In addition, although, the fact that darifenacin is a lipophilic compound and is a substrate for P-glycoprotein, so in theory it should have been carried via the BBB, however, according to Skerjanec (2006) darifenacin can penetrate the BBB and to confirm this (Skerjanec, 2006), results from the PET and audioradiographic studies by Yoshida and colleagues (2010) show that darifenacin has a tendency to bind to muscarinic receptors located in the CNS, although not as effectively when compared with other centrally acting M3 receptor antagonist (Yoshida *et al.*, 2010).

In contrast to M2 and M3 antagonists, relatively M1 and M4 selective compounds showed the highest potency in inhibition of pilocarpine-induced purposeless chewing. These findings are not unexpected and are consistent with the observation that both subtypes of receptors are the most abundantly expressed in the striatum and play a role in motor control (Waelbroeck *et al.*, 1990; Eglen, 2006; Langmead *et al.*, 2008).

Based on obtained results, systemic administration of centrally acting relatively M1 selective benztropine and trihexyphenidyl, nearly completely inhibited pilocarpine-induced purposeless chewing in a dose dependent manner. These findings are in accordance with previously published studies, where these compounds showed similar pattern of effect on inhibition of chewing (Salamone *et al.*, 1986; Stewart *et al.*, 1988; Stewart *et al.*, 1989). In addition, both benztropine and trihexyphenidyl are commonly used clinically to aid the treatment of neurologic disorders such as dystonia, PD, tremor or drug-induced movement disorders (Pidcock *et al.*, 1999; Jankovic, 2006). Moreover, recent results from preclinical investigations suggest that selective antagonism of muscarinic M1 receptors could be useful in the treatment of dystonia or PD (Xiang *et al.*, 2012; Erosa-Rivero *et al.*, 2014).

Similarly, to M1 antagonists, M4 relative selective compounds also had an inhibitory effect on purposeless chewing. Results show that tropicamide, partially selective M4 antagonist, was effective and significantly inhibited pilocarpine-induced purposeless chewing which was seen in a dose-dependent manner. These data support findings by Betz *et al.,* (2007) who also used tropicamide as the only moderately selective M4 antagonist which readily crosses BBB, and demonstrated antiparkinsonian properties of this compound. Based on results from current and previous study, since tropicamide shows only moderate selectivity for M4 receptors, further examination with highly selective M4 compounds was essential to elucidate whether these effects are indeed due to the M4 receptors. Therefore, the clinical need for a selective M4 antagonist has led to the development of the novel highly selective NBI-675.

As anticipated, NBI-675 showed significant inhibition of pilocarpine-induced purposeless chewing in a dose-dependent manner. Nevertheless, its effects were similar to the effects of tropicamide, which was unforeseen, considering the fact that the novel compound shows 46-fold higher selectivity for M4 receptors than tropicamide, when tested *in vitro* (Chapter 1 Table 1.5 of pKi). A possible reason for

this could be caused by differences in PK, drug stability, its effect on the organism, BBB penetration or as mentioned earlier, reduction of selectivity with increase of dose or other yet unknown parameters that affect drug activity *in vivo.*

3.4.2 The effect of centrally administered anticholinergics

As mentioned earlier, pirenzepine is a hydrophilic compound and does not cross the BBB (Carmine & Brogden, 1985), therefore to test its central effects on pilocarpine-induced purposeless chewing, pirenzepine was administered i.c.v. Pirenzepine nearly completely suppressed pilocarpine-induced chewing, which was seen in a dose-dependent manner and this again shows and supports the findings that purposeless chewing is centrally mediated. These results are in accordance with the study by Mayorga *et al.,* (1999) where pirenzepine was seen to inhibit chewing in a dose-dependent fashion.

Interestingly, other drugs administered via central route do not show such strong inhibition. Intracerebroventricular (i.c.v.) administration of trihexyphenidyl only partially suppressed purposeless chewing, and this result was unforeseen, as peripheral administration of the drug showed strong inhibitory properties. In addition, small inhibition of chewing was observed after central injection of darifenacin. This effect was not unexpected, since darifenacin is a M3 selective antimuscarinic and according to the evidence, high levels of M3 receptors are located in the periphery (Eglen 2006; Langmead *et al.*, 2008). Lastly, tropicamide had no inhibitory effect on pilocarpine-induced chewing after central administration.

It is highly probable that inability of centrally administered antagonists, except of pirenzepine, was caused by limited drug solubility and, thus, the higher doses could not be tested.

Overall, based on the results obtained in this chapter, selective M1 and M4 muscarinic antagonists showed the most potent inhibitory properties of pilocarpine-induced chewing. As mentioned earlier (Chapter 1, section 1.3.2.2) both M1 and M4 subtypes of muscarinic receptors are highly expressed in the striatum, where they play different roles including regulation of motor control (M1 and M4). M1 subtypes also have a function in memory control and attention and in addition, they are expressed in the periphery, mainly in the glands. Thus, since both types of these receptors are present in the striatum, which comprises connection of different nuclei involved, among

others, in motor planning and execution, there is a possibility that both subtypes might be responsible for the motor movement (Graybiel *et al.*, 1994; Ponterio *et al.*, 2012).

In addition, these findings agree with the studies on muscarinic receptors knock out mice, which indicated that absence of M1 and M4 receptors improves locomotor activity. Behavioural tests, including elevated plus maze, light/dark transition test, open field test in M1 receptor deficient mice ($M1R^{-/-}$) showed that the $M1R^{-/-}$ were more hyperactive than their wild type (WT) littermates (Miyakawa *et al.*, 2001). Similarly, deactivation of M4 receptors in mice ($M4R^{-/-}$) led to increase in basal locomotor activity in $M4R^{-/-}$ when compared to the control littermates, as it was shown by an interruption of photo-beams in an open field test (Gomeza *et al.*, 1999b). By contrast, M2, M3 and M5 receptor knock out mice showed comparable locomotor responses when compared with their WT littermates (Gomeza *et al.*, 1999a; Yamada *et al.*, 2001a; Yamada *et al.*, 2001b).

3.5. Conclusion

In conclusion, this study confirms involvement of central muscarinic receptors in production of purposeless chewing in rats. Majority of all centrally acting anticholinergics tested, after systemic administration, showed inhibitory effect on pilocarpine-induced purposeless chewing. However, it is hard to conclude the exact muscarinic receptor subtype involved in mediation of purposeless chewing. Muscarinic M1 receptors play a role in pilocarpine-induced purposeless chewing, as central administration of pirenzepine nearly completely suppressed chewing. Similarly, benztropine and trihexyphenidyl inhibited chewing when administered systemically. AF-DX 116 only partially reduced purposeless chewing induced by pilocarpine suggesting, limited involvement of M2 receptors. Likewise, there is little contribution in mediation of purposeless chewing by M3 receptors, since darifenacin has shown little effect on suppression of pilocarpine-induced purposeless chewing. Nevertheless, clearly there is a role of M4 receptors in pilocarpine-induced purposeless chewing, as both tropicamide and NBI-675 showed highly antagonistic effect on this chewing behaviour. This suggests that our hypothesis that centrally mediated motor dysfunction (abnormal involuntary movements) is supressed by anticholinergics through inhibition of muscarinic M4 receptors can be accepted.

Overall, compounds showing the highest potency in inhibition of pilocarpine-induced purposeless chewing are selective for M1 and M4 receptor, indicating that selective inhibition of these receptors may be of therapeutic benefit in management of locomotor activity and coordination. However, involvement of M1 receptors provides huge disadvantage for clinical use, since these receptors are located in the periphery, as well as the CNS (Eglen 2006; Langmead *et al.,* 2008), thus increasing the risk of side effects, which have been well documented as a limiting factor in the treatment of movement disorders, in particular, oral dryness (Jankovic, 2006; Cloud & Jinnah, 2010; Lubarr & Bressman, 2011). Therefore, the studies in the following chapter will focus on investigation of the effect of anticholinergics on pilocarpine-induced saliva secretion in rats, in order to determine if selective suppression of muscarinic receptor M1 and M4 receptors would provide aid in the treatment of dystonia without inducing dry mouth.

Chapter 4 The effect of anticholinergics treatment on pilocarpine-induced saliva secretion

4.1. Introduction

In the preceding chapter, it was reported that anticholinergics have a differential inhibitory effect on pilocarpine-induced purposeless chewing in rats and it was concluded that centrally located muscarinic M1 and M4 receptors are largely responsible for this effect. From this study, it was suggested that M1 or M4 receptor subtypes located in the striatum could be beneficial in the management of dystonia.

Currently, anticholinergics are effectively used in the treatment of dystonia, but their use is limited due to their unpleasant centrally and peripherally mediated side effects, in particular the impairment in salivary flow resulting in a dry mouth (Cloud & Jinnah, 2010; Lubarr & Bressman, 2011). For many patients, oral dryness (xerostomia) is often a limiting factor of anticholinergic therapy and in many cases is uncomfortable and causes not only difficulties related to ingestion of food and swallowing but also prevents speech (Wiseman & Faulds, 1995) and can lead to oral health complications, such as dental caries or candidiasis. All these can have a negative impact on quality of life (Wiseman & Faulds, 1995; Sreebny & Schwartz, 1997).

Pilocarpine, as a non-selective muscarinic agonist, not only acts on central muscarinic receptors to produce purposeless chewing in rats, as described in Chapter 3, but also, by acting on ACh receptors in the salivary glands, stimulates saliva secretion. Indeed, for this reason, pilocarpine is commonly used for the treatment of oral dryness in patient that suffer from dysfunction of salivary gland (Ferguson, 1993; Wiseman & Faulds, 1995; Fox *et al.*, 2001). However, for the purpose of this study, pilocarpine can be used to test the properties of antagonists to induce oral dryness, by oral introduction of a pre-weighted swab into rats cheek (Flynn *et al.*, 1980).

Evidence from rat studies show that salivation is mediated mainly by muscarinic M3 receptors (Baum, 1993; Gautam *et al.*, 2004; Proctor & Carpenter, 2007). However, other studies suggest partial involvement of M1 receptors in addition to M3 (Culp *et al.*, 1991; Tobin, 1995; Tobin *et al.*, 2002) and some propose that M5 receptors may also play a role (Gautam *et al.*, 2004).

In light of the results presented in the previous chapter, it was suggested that central M1 and M4 receptor subtypes play a role in mediation of abnormal involuntary movements resulting in dystonia. Importantly, with respect to potential side effects of antagonists at these receptors, muscarinic M1 receptors are not only present in the

brain, including striatum, but also the periphery, where they regulate exocrine secretion, whereas M4 receptors are largely expressed in the CNS, suggesting limited propensity to elicit these peripheral effects. For this reason, it is important to investigate the involvement of these receptors in the mediation of peripheral side effects and in particular salivation, and this form the basis of current investigation (Waelbroeck *et al.*, 1990; Eglen, 2006; Langmead *et al.*, 2008).

4.1.1. Hypothesis

It is hypothesised that selective antagonism of muscarinic M4 receptors which reduce dystonia will not induce unfavourable oral dryness.

4.1.2. Aims

Specific aims of this study were to:

1) Use pilocarpine to induce salivation in rats and to compare the ability of non-selective and subtype selective anticholinergics to inhibit pilocarpine-induced salivation.
2) Confirm that peripheral muscarinic receptors are responsible for pilocarpine-induced saliva secretion.
3) Confirm the muscarinic receptor subtype responsible for mediation of salivation.
4) Determine the role of M4 receptors on pilocarpine-induced salivation using a novel selective muscarinic M4 antagonist, NBI-675.

4.2 Materials and Methods

In order to address these aims the following studies were performed:

1) The role of peripheral muscarinic receptors in mediation of pilocarpine-induced saliva secretion was confirmed by treating rats with peripherally and centrally acting anticholinergics 30 min prior to pilocarpine. Saliva secretion was measured for up to 120 min.
2) In order to confirm subtype of muscarinic receptor involved in mediation of pilocarpine-induced salivation, rats were treated with relatively selective anticholinergics 30 min prior pilocarpine and the saliva secretion was measured for up to 120 min.
3) The role of the M4 muscarinic receptor in pilocarpine-induced salivation was determined using the novel selective M4 antagonist NBI-675. NBI-675 was administered 60 min prior to pilocarpine, and saliva secretion was measured as described below.

Detailed methodology is described below.

4.2.1 Animal husbandry

Male Wistar rats (160 – 300 g, Harlan, UK) were housed in groups of 4 – 5 per cage at a temperature of 22 ± 2ºC and humidity controlled housing unit on a 12 hours light – dark cycle. Water and food were available *ad libitum* except during the surgery and experiments. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 under UK Home Office Project licence number 70/6898 and 70/7977, approved by the King's College London Ethical Review Panel.

4.2.2 Assessment of saliva secretion

As described in Chapter 2, section 2.2.2, systemic administration of pilocarpine stimulates saliva secretion. This can be suppressed by the administration of anticholinergics.

Animals were placed in a Perspex observation boxes (21 x 35 x 17 cm) and pilocarpine (3.4 mg/kg i.p.) was administered 30 min after anticholinergics (i.p. or i.c.v.) administration. Measurement of salivation was conducted 10 min before administration of anticholinergics (time 0, baseline), 10 min before and 20, 60, 90 and 120 min after pilocarpine administration.

Measurement of salivation was conducted by inserting a pre-weighted cotton tipped swab into the rat's cheek for 10 s and reweighing it. The saliva weight was calculated by subtracting the initial from final weight of the swab. Detailed methodology is described in Chapter 2 section 2.2.2.1.

4.2.3 Drug treatment

4.2.3.1 Peripheral administration of anticholinergics

Trihexyphenidyl hydrochloride (0.001 – 5 mg/kg), benztropine mesylate (0.001 – 5 mg/kg), darifenacin hydrobromide (0.001 – 24 mg/kg), tropicamide (0.001 – 20 mg/kg), AF-DX 116 (0.1 – 30 mg/kg), scopolamine hydrobromide (0.01 – 1 mg/kg), methylscopolamine (0.01 – 1 mg/kg) and NBI-675 (0.1 – 30 mg/kg) were administered i.p. 30 min prior pilocarpine (3.4 mg/kg i.p.). Additionally, NBI-675 (0.1 – 30 mg/kg) was administered 1 hr prior pilocarpine by oral gavage (p.o.). Doses of the drugs were chosen according to the ID_{50} doses for inhibition of pilocarpine-induced purposeless chewing described in the previous chapter of this thesis, based on previously published studies (Stewart *et al.*, 1989; Mayorga *et al.*, 1999; Betz *et al.*, 2007) and according to data from the PK and PD studies (NBI-675) received from Neurocrine Biosciences Inc.

Trihexyphenidyl was dissolved in deionised water, darifenacin and AF-DX 116 were dissolved in a sterile dimethyl sulphoxide (DMSO) and diluted in 0.9% saline (for darifenacin 5:1 saline to DMSO, and for AF-DX 116 3:1 saline to DMSO) and tropicamide was dissolved in a minimal amount of ethanol (200 µl) and diluted in 0.9% saline. NBI-675 was dissolved in 5% Tween80 and 0.5% methylcellulose made up in deionised water, vortexed and sonicated for 1 hr. The pH was adjusted to 4 – 6 with 1M NaOH. All other drugs were dissolved in 0.9% saline and administered intraperitoneally at a dose volume of 1 ml/kg, except the highest dose of AF-DX 116 (30 mg/kg) which, due to the problems with solubility, was administered at a dose volume of 2 ml/kg.

Rats were placed in observation boxes and measurement of salivation was conducted as described in section 2.2.2.1. Drug treatments were administered following a crossover design, so each rat was treated with all doses of a single drug or vehicle with at least 48 hr between the tests. A typical modified latin square was used to randomise

drug treatments, and is shown in Table 3.1 Section 3.2.3.1. Individual anticholinergics were tested in separate groups of animals.

4.2.3.2 Central administration of anticholinergics

4.2.3.2.1 Stereotaxic guide cannula implantation into the lateral ventricle

A stainless steel guide cannula (23 G) was implanted into the lateral ventricle (AP: -0.8 mm; ML: -1.4 mm; DV: -2.8 mm) using standard stereotaxic techniques as described in Chapter 3, Section 3.2.3.2.1.

The post-operative weight was monitored daily and a mash diet (softened food pellets) provided until animals regained their pre-operative weight, as shown in Figure 3.3, Section 3.2.3.2.1. Animals were allowed 6 days to recover prior behavioural testing.

4.2.3.2.2 Intracerebroventricular drug administration

Pirenzepine dihydrochloride (2.4 – 377 nmol/µl i.c.v., equivalent of 1 – 160 µg/µl) was dissolved in 0.9% saline and, due to limited solubility, administered in a dose volume of 2 µl and 3 µl (the highest dose) at a rate 1 µl/min. Darifenacin hydrobromide (0.2 – 98.5 nmol/µl, equivalent of 0.1 – 50 µg/µl) was dissolved in a sterile DMSO and diluted with 0.9% saline (4:1 saline to DMSO) and administered i.c.v. at a dose volume of 2 µl at a rate 1 µl/min. Trihexyphenidyl (3 – 59 nmol, equivalent of 1 – 20 µg/µl) was dissolved in a deionised water and administered i.c.v. at a dose volume of 1 µl (3 nmol), 2 µl (29.5 nmol) and 4 µl (59 nmol), due to the poor solubility, at a rate 1 µl/min. Tropicamide (3.5 – 422 nmol/ µl, equivalent of 1 – 120 µg/µl) was dissolved in a minimal amount of ethanol (20 µl) and diluted in 0.9% saline and administered i.c.v. at a dose volume of 2 µl at a rate 1 µl/min.

Drug or vehicle was administered into the lateral ventricle via previously implanted guide cannula (described in Section 3.2.3.2.1) 30 min before pilocarpine. Microinjection was made via stainless steel injection needle set to extend 1 mm beyond the tip of the guide cannula implanted previously into right lateral ventricle. Detailed methodology is described in Chapter 3, section 3.2.3.2.1. Rats were placed in boxes and measurement of salivation was conducted as described in section 2.2.4.1. Drug treatments were administered on a crossover design, so each rat was treated with all

doses of a single drug or vehicle at least 48 hr between the tests (Table 3.1 Section 3.2.3.1). Drugs were tested individually in separate studies.

4.2.4 Statistical analysis

Data and statistical analysis were performed using GraphPad Prism 5.02 (San Diego, CA, USA). Area under curve (AUC) for the time course was calculated by trapezoid method. Data are expressed as mean ± SEM and analysed by sigmoidal nonlinear regression analysis 3-parameter fit. ID_{50} value was derived from the curve fit. The basal salivation levels within 20 min of antagonist administration and the differences between treatment and vehicle control (mean for both vehicle treatment before pilocarpine administration) for AUC negative peaks data was analysed by One-way ANOVA followed by a *post hoc* Dunnett's Multiple Comparison test. Differences between treatment and vehicle control for AUC data was analysed by One-way ANOVA followed by a *post hoc* Dunnett's Multiple Comparison test.

4.3. Results

The IC_{50} dose of pilocarpine, 3.4 mg/kg i.p., was chosen according to previous studies (Chapter 2 section 2.2.1.4) to induce saliva secretion in rats. As expected, this dose of pilocarpine stimulated salivary secretion, which was visible within few minutes, with a peak about 20 min of administration (Figs. 4.1 – 4.13). Administration of vehicle did not have effect on saliva secretion (Figs. 4.1 – 4.13).

In order to confirm muscarinic receptor subtype responsible for mediation of saliva secretion, the effect of non-selective and subtype selective anticholinergics was investigated on pilocarpine-induced saliva secretion.

4.3.1 The effect of non-selective anticholinergics on pilocarpine-induced salivation

Scopolamine (0.01 – 1 mg/kg), a centrally and peripherally acting non-selective anticholinergic, produced a small, dose-related, reduction in basal salivation, which was significant at 20 min following 0.1 and 1 mg/kg when compared to vehicle-treated animals (Fig. 4.1 C).

Following administration of pilocarpine, the lowest dose of scopolamine (0.1 mg/kg) had no effect on suppression of pilocarpine-induced salivation, while the highest dose (1 mg/kg) produced about 71% inhibition of pilocarpine-induced saliva secretion, which was significantly different when compared to vehicle-treated animals (Fig. 4.1 A & B). The ID_{50} for scopolamine was 0.25 mg/kg (95% CI = 0.05 – 1.2 mg/kg).

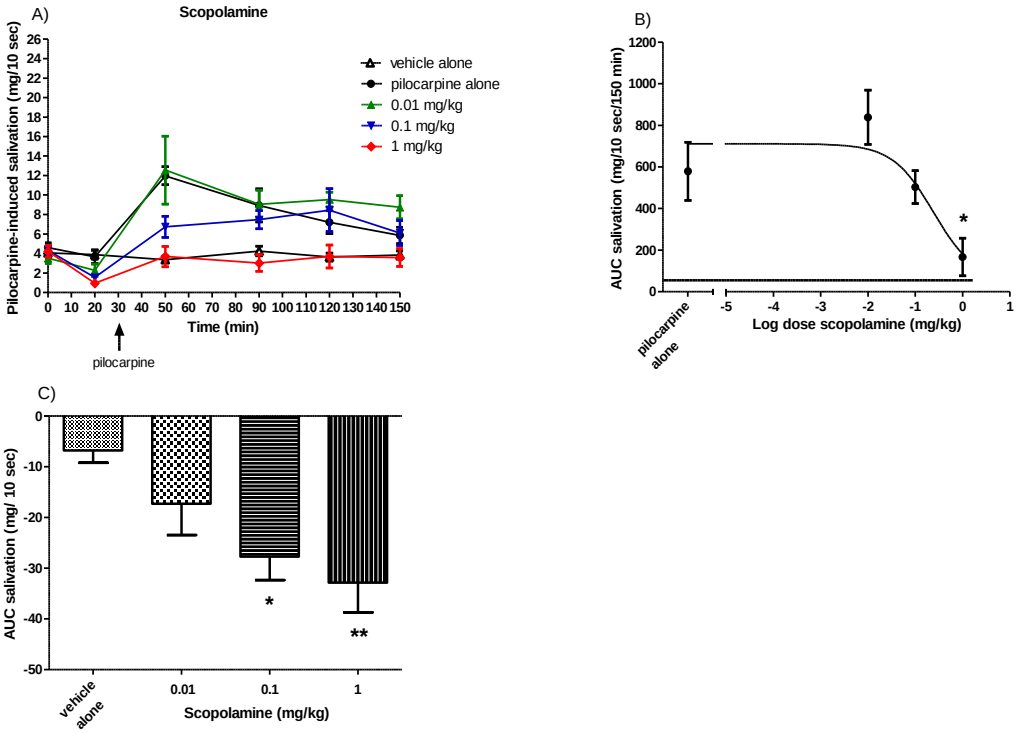


Figure 4.1 Effect of scopolamine (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for scopolamine (0.01 – 1 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). Estimated ID_{50} = 0.25 mg/kg; (95% CI = 0.05 – 1.2 mg/kg; r^2 = 0.35, NS); *p<0.05 compared to vehicle-treated animals (One way ANOVA (p<0.01; F = 6.002; Df = 3, 24) followed by Dunnett's Multiple Comparison test). C) Effect of scopolamine on saliva within the first 20 min (AUC negative peaks time course). *p<0.05; ** p<0.01 (One way ANOVA (p<0.01; F = 5.302; Df = 3, 24) followed by Dunnett's Multiple Comparison test).

Administration of methylscopolamine (0.01 – 1 mg/kg), the peripherally acting non-selective muscarinic antagonist, also reduced basal salivation within the first 20 min, which was significantly different when compared to vehicle-treated animals at the highest dose (Fig. 4.2 C).

Methylscopolamine produced a dose-related reduction of pilocarpine-induced salivation, with a significant effect at the two highest doses (0.1 and 1 mg/kg) when compared to vehicle-treated animals (Fig. 4.2 A & B). Indeed, the highest dose tested (1 mg/kg) resulted in a complete inhibition of pilocarpine-induced saliva secretion,

and in fact tended to reduce salivation to below baseline values (Fig 4.2 B). The ID_{50} for methylscopolamine was 0.024 mg/kg (95% CI = 0.004 – 0.5 mg/kg).

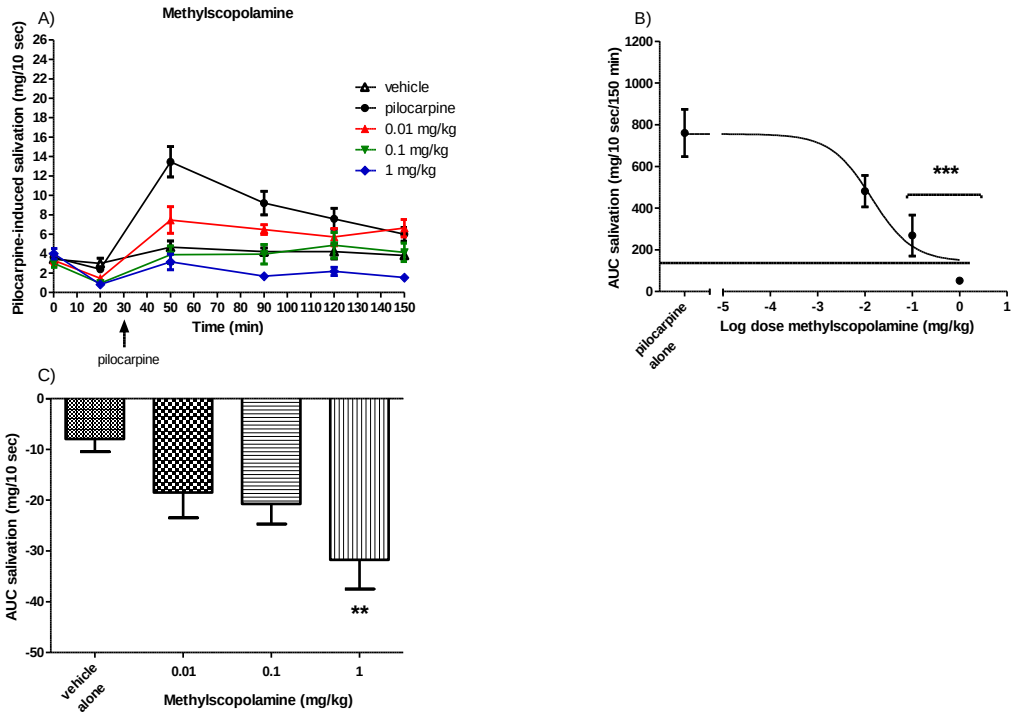


Figure 4.2 Effect of methylscopolamine (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for methylscopolamine (0.01 – 1 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} = 0.02 mg/kg; (95% CI = 0.004 – 0.5 mg/kg; r^2 = 0.55 *p<0.05; ***p<0.001 compared to vehicle-treated animals (One way ANOVA (p<0.001; F = 12.89; Df = 3, 28) followed by Dunnett's Multiple Comparison test). C) Effect of methylscopolamine on saliva within the first 20 min (AUC negative peaks time course). **p<0.01 (One way ANOVA (p<0.01; F =4.784; Df = 3, 28) followed by Dunnett's Multiple Comparison test).

4.3.2 The effect of subtype selective anticholinergics

4.3.2.1 Muscarinic M1 selective anticholinergics

Treatment with the M1 selective antagonist, trihexyphenidyl (0.001 – 5 mg/kg i.p.), had no effect on basal salivation during the first 20 minutes of administration (Fig. 4.3 C).

Following administration of pilocarpine, trihexyphenidyl produced a small, non-dose-related reduction of pilocarpine-induced salivation (Fig. 4.3 A & B). The effect was significantly reduced at the doses of 0.01, 1.5 and 5 mg/kg when compared to vehicle-treated animals (Fig. 4.3 B). The highest dose tested showed about 46 % of inhibition. As the effect was not dose-related (r^2 = 0.06; NS), the ID_{50} could not be determined.

Central administration of trihexyphenidyl (3 – 59 nmol i.c.v., equivalent to 1, 10 and 20 µg) into the lateral ventricle had no effect on basal salivation within the 20 min of administration (Fig. 4.4 C), and no effect on pilocarpine-induced salivation (Fig. 4.4 A & B). Due to the lack of significant inhibition, the ID_{50} could not be obtained.

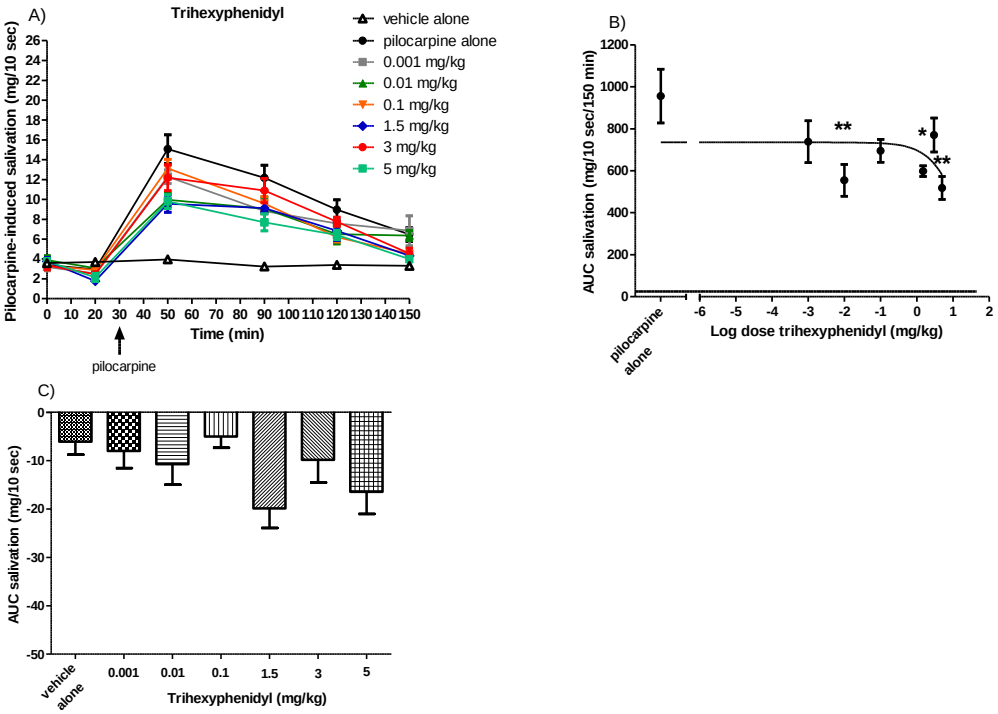


Figure 4.3 Effect of trihexyphenidyl (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for trihexyphenidyl (0.001 – 5 mg/kg i.p) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} > 5 mg/kg; (r^2 = 0.06 NS); *p<0.05; **p<0.01 compared to vehicle-treated animals (One way ANOVA (p<0.01; F = 3.501; Df = 6, 42) followed by Dunnett's Multiple Comparison test). C) Effect of trihexyphenidyl on saliva within the first 20 min (AUC negative peaks time course). NS (One way ANOVA (p=0.0859; F = 2.008; Df = 6, 42)).

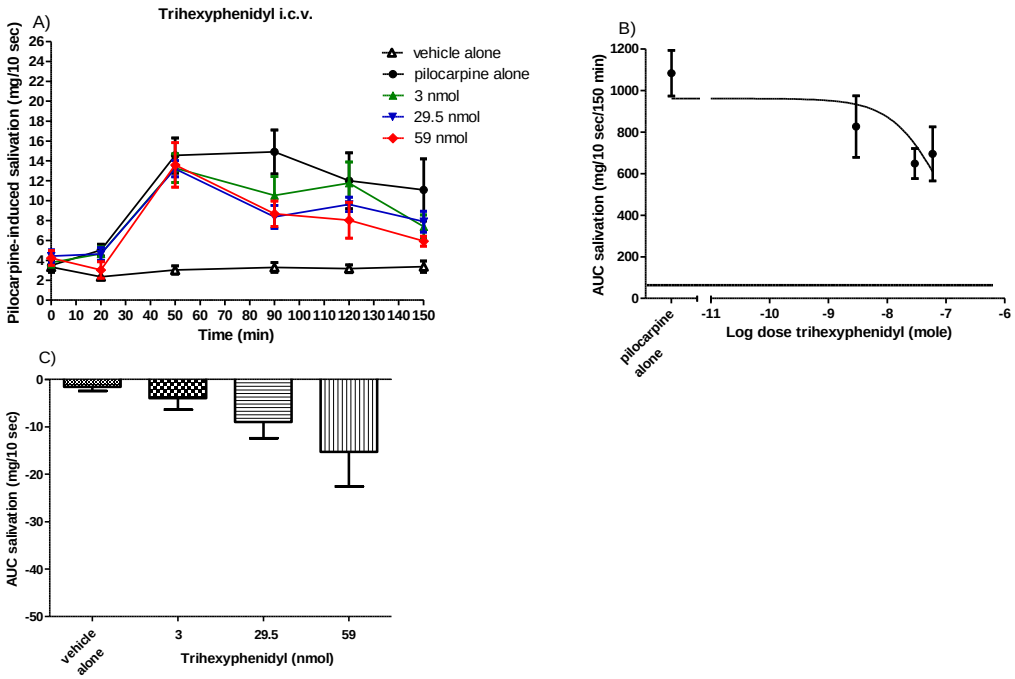


Figure 4.4 Effect of trihexyphenidyl (i.c.v.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for trihexyphenidyl (3 – 59 nmol i.c.v., equivalent to 1 – 20 µg) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} > 59 nmol; (r^2 = 0.26; NS); NS compared to vehicle-treated animals (One way ANOVA (p=0.0671; F = 2.716; Df = 3, 24). C) Effect of trihexyphenidyl on saliva within the first 20 min (AUC negative peaks time course). NS (One way ANOVA (p=0.1330; F = 2.055; Df = 3, 24)).

The M1 antagonist benztropine (0.001 – 5 mg/kg i.p.), had no effect on basal salivation levels within 20 min of administration (Fig. 4.5 C).

Following pilocarpine administration, benztropine had little or no effect on inhibition of pilocarpine-induced saliva secretion at lower doses. The highest dose (5 mg/kg) tended to reduce pilocarpine-induced salivation, however, this was not significant when compared to vehicle-treated animals (Fig. 4.5 B). The ID_{50} was greater than 5 mg/kg, the highest dose administered.

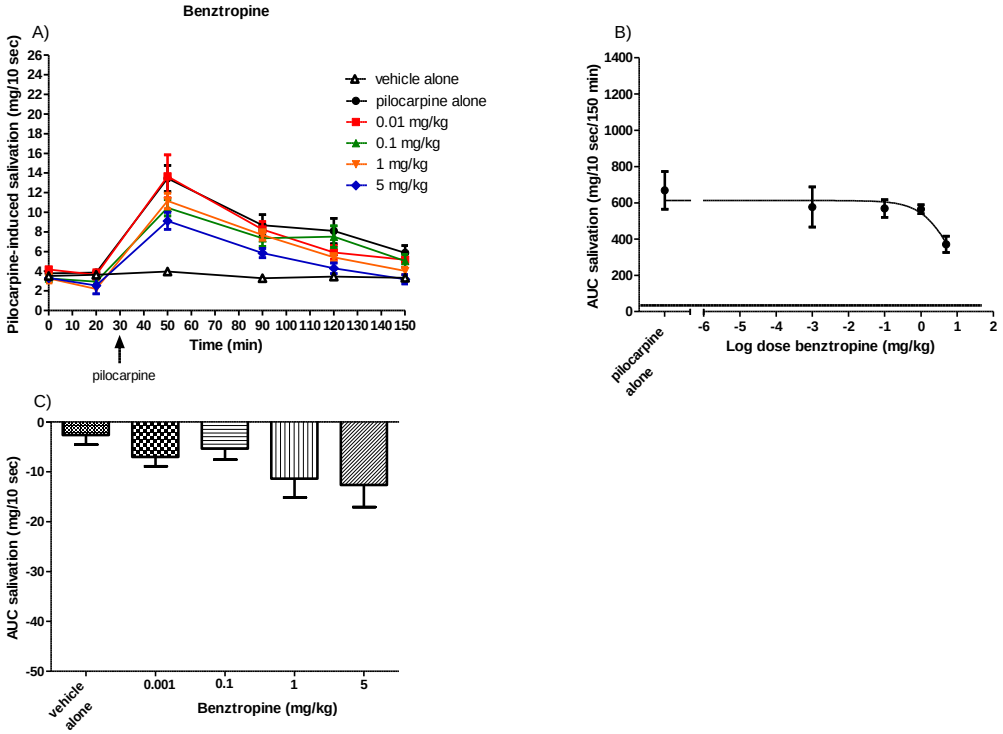


Figure 4.5 Effect of benztropine (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for benztropine (0.001 – 5 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit. ID_{50} > 5 mg/kg; the bottom was constrained to the mean of vehicle alone values (black line). NS compared to vehicle-treated animals (One way ANOVA (p=0.1002; F = 2.111; Df = 4, 35)). C) Effect of benztropine on saliva within the first 20 min (AUC negative peaks time course). NS (One way ANOVA (p=0.1354; F = 1.883; Df = 4, 35)).

Systemic administration of pirenzepine, the selective M1 antagonist, reduced basal salivation levels within 20 min of administration at all doses tested (0.1 – 50 mg/kg i.p.), with significant effect at 30 and 50 mg/kg when compared to vehicle-treated animals (Fig. 4.6 C).

Pirenzepine dose-dependently reduced secretion of saliva induced by pilocarpine with significant effect at the doses 30 and 50 mg/kg when compared to vehicle-treated animals (Fig. 4.6 A & B). The two highest doses resulted in a complete suppression of salivation with the values reaching basal levels (Fig. 4.6 B). The ID_{50} for pirenzepine was 4.9 mg/kg (95% CI = 1.6 – 14.98 mg/kg).

Central administration pirenzepine (2.4 – 377 nmol i.c.v., equivalent to 1 – 160 µg) produced a dose-related reduction of basal salivation levels with significant effect at

189 and 377 nmol during the first 20 min when compared to vehicle-treated animals (Fig. 4.7 C).

Intracerebroventricular administration of pirenzepine did not reduce pilocarpine-induced salivation however, the higher doses (47 – 377 nmol) resulted in increased secretion of saliva, which was significantly different for the two high doses (189 and 377 nmol) when compared to vehicle-treated animals (Fig. 4.7 A & B). The ID_{50} could not be determined.

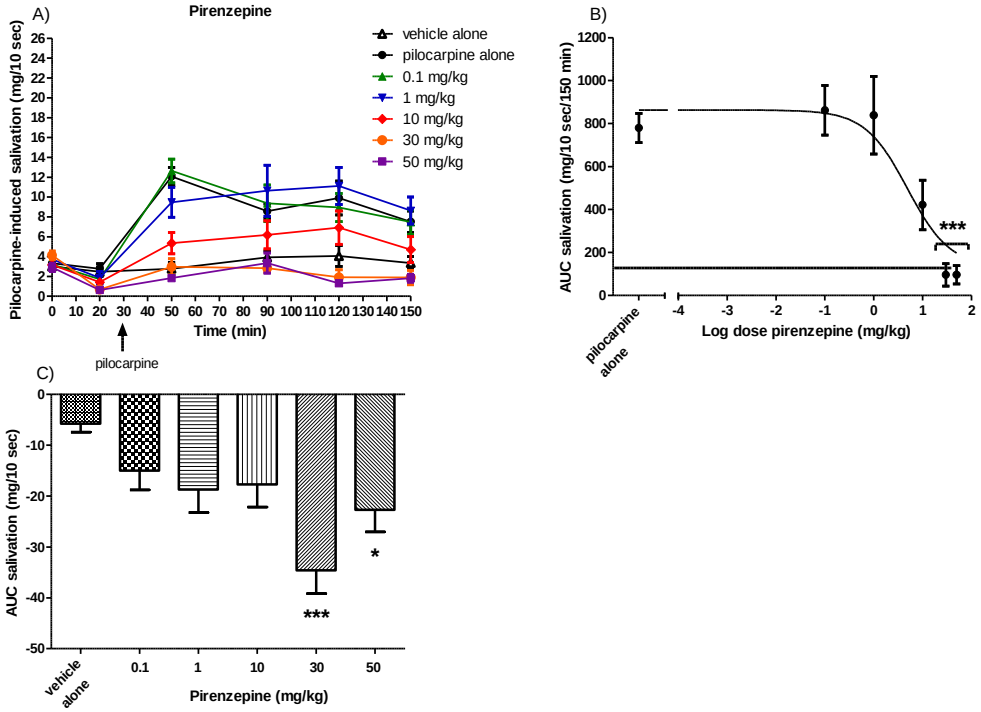


Figure 4.6 Effect of pirenzepine (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for pirenzepine (0.1 – 50 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} = 4.9 mg/kg ± 1.74 mg/kg; (95 % CI = 1.6 – 14.98; r^2 = 0.57; *p<0.05) ***p<0.001 compared to vehicle-treated animals (One way ANOVA (p<0.001; F = 11.45; Df = 5, 36) followed by Dunnett's Multiple Comparison test). C) Effect of pirenzepine on saliva within the first 20 min (AUC negative peaks time course). *p<0.05; *** p<0.001 (One way ANOVA (p<0.001; F = 5.565; Df = 5, 36) followed by Dunnett's Multiple Comparison test).

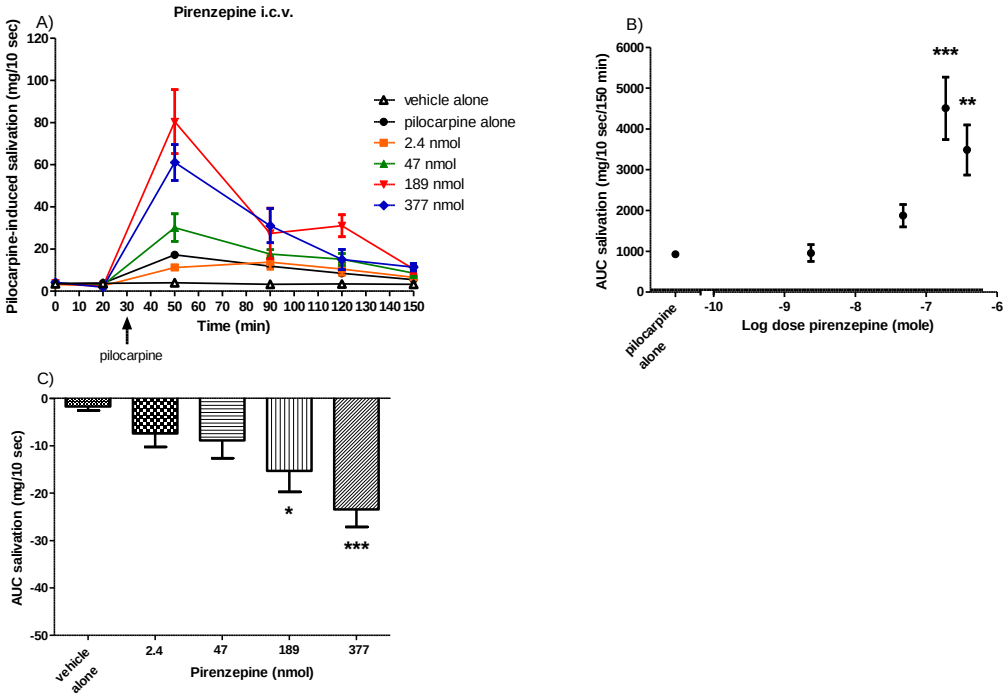


Figure 4.7 Effect of pirenzepine (i.c.v.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for pirenzepine (2.4 – 377 nmol i.c.v., equivalent to 1 – 160 µg) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit, not converged); **p<0.01; ***p<0.001 compared to vehicle-treated animals (One way ANOVA (p<0.001; F = 11.70; Df = 4, 30) followed by Dunnett's Multiple Comparison test). C) Effect of pirenzepine on saliva within the first 20 min (AUC negative peaks time course). *p<0.05; ***p<0.001 (One way ANOVA (p<0.01; F = 6.090; Df = 4, 30) followed by Dunnett's Multiple Comparison test).

4.3.2.2 Muscarinic M2 selective anticholinergics

The M2 antagonist AF-DX 116 (0.1 – 30 mg/kg i.p.) had no effect on basal salivation levels (Fig. 5.8 C) and no significant effect on pilocarpine-induced salivation (Fig. 5.8 A & B). The ID_{50} could not be determined.

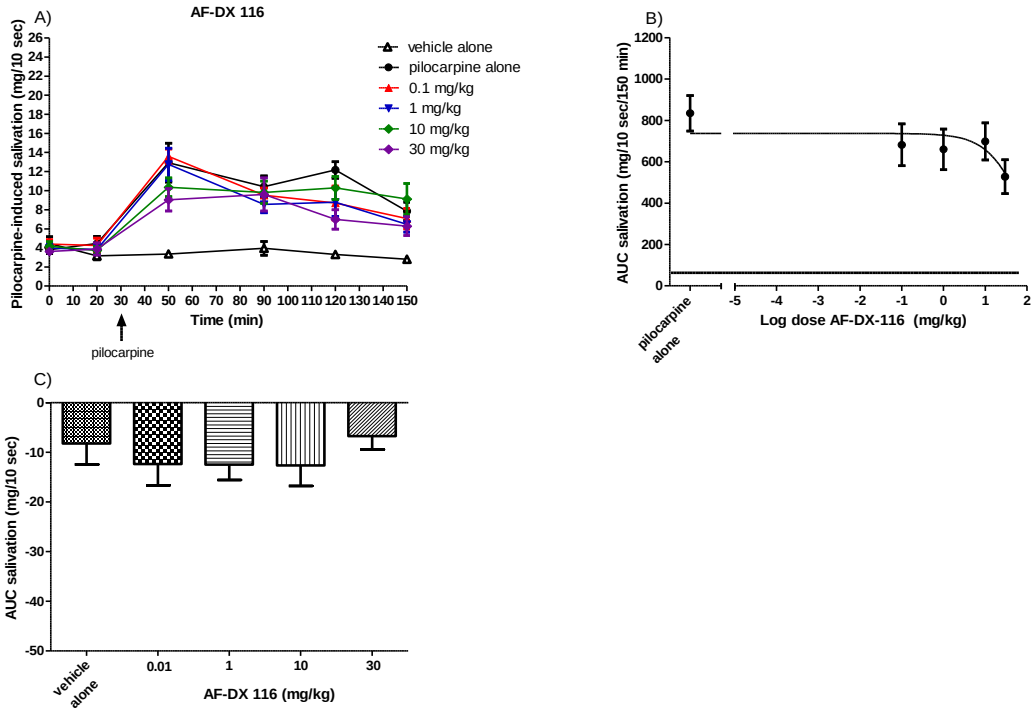


Figure 4.8 Effect of AF-DX 116 (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for AF-DX 116 (1 – 30 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} > 30 mg/kg; (r^2 = 0.08 NS); NS compared to vehicle-treated animals (One way ANOVA (p=0.2496; F = 1.414; Df = 4, 35)). C) Effect of AF-DX 116 on saliva within the first 20 min (AUC negative peaks time course). NS (One way ANOVA, p=0.6941; F =0.5586; Df = 4, 35)).

4.3.2.3 Muscarinic M3 selective anticholinergics

Administration of darifenacin (0.001 – 24 mg/kg i.p.), M3 selective antagonist, tended to show a dose-related inhibition of basal salivation, although the effect was not significant during the first 20 min when compared to vehicle-treated animals.

Darifenacin dose-dependently inhibited pilocarpine-induced saliva secretion (Fig.4.9 A) with significant effect at doses 0.1 – 24 mg/kg when compared to vehicle-treated animals (Fig. 4.9 B). The highest tested dose resulted in 96% of suppression of pilocarpine-induced salivation. The ID_{50} for darifenacin was 0.4 mg/kg (95% CI = 0.2 – 0.9 mg/kg).

Central administration of darifenacin (0.2 – 98.5 nmol i.c.v., equivalent to 0.1 – 50 µg) into the lateral ventricle tended to reduce basal salivation levels within 20 min of administration, although this effect was not dose related or statistically significant.

Darifenacin had no significant effect on pilocarpine-induced saliva secretion (Fig. 4.10 A). Due to the lack of effect the ID_{50} could not be determined.

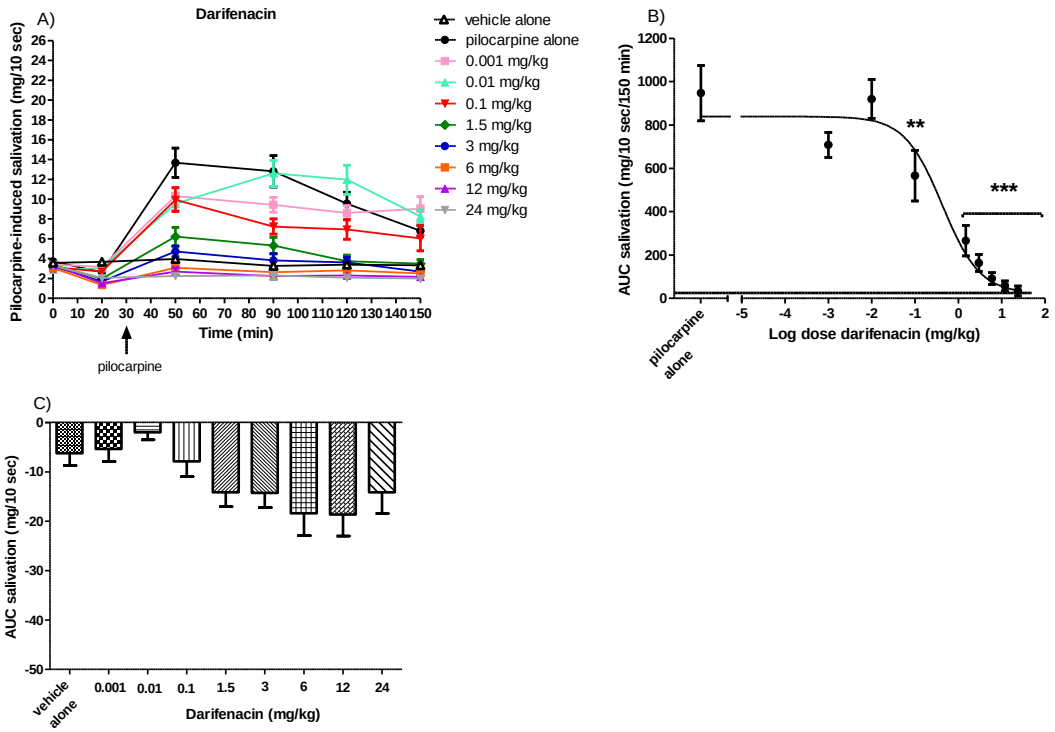


Figure 4.9 Effect of darifenacin (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 8); data were normalised to the baseline to calculate AUC. B) Log-dose response for darifenacin (0.001 – 24 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} = 0.4 ± 1.5 mg/kg; (95% CI = 0.2 – 0.9 mg/kg; r^2 = 0.72, $p<0.05$); **$p<0.01$; ***$p<0.001$ compared to vehicle-treated animals (One way ANOVA ($p<0.001$; F = 25.15; Df = 8, 63) followed by Dunnett's Multiple Comparison test). C) Effect of darifenacin on saliva within the first 20 min (AUC negative peaks time course). NS (One way ANOVA ($p<0.01$; F = 3.240; Df = 8, 63) followed by Dunnett's Multiple Comparison test).

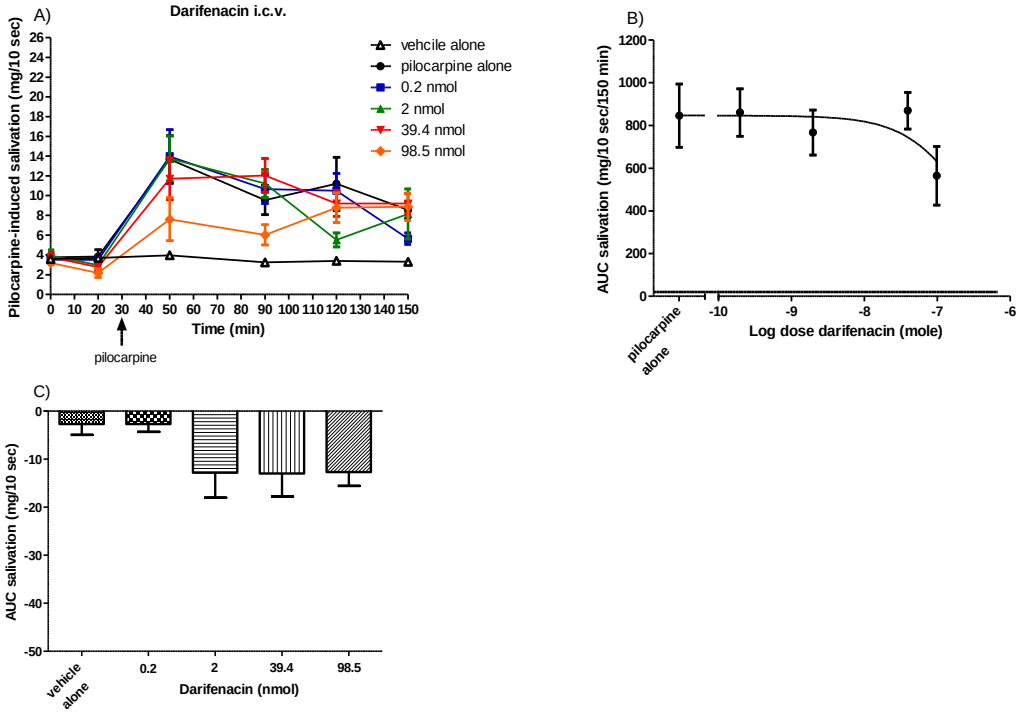


Figure 4.10 Effect of darifenacin (i.c.v.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for darifenacin (0.2 – 98.5 nmol i.c.v., equivalent to 0.1 – 50 µg) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} > 98.5 nmol; NS compared to vehicle-treated animals (One way ANOVA (p=0.3546; F = 1.145; Df = 4, 30)). C) Effect of darifenacin on saliva within the first 20 min (AUC negative peaks time course). NS (One way ANOVA (p=0.0771; F = 2.346; Df = 4, 30)).

4.3.2.4 Muscarinic M4 selective anticholinergics

The putative, relatively selective M4 antagonist, tropicamide (0.001 – 20 mg/kg) alone showed significant effect on suppression of basal salivation at doses 0.01 and 0.6 – 20 mg/kg within 20 min of administration when compared to vehicle-treated animals (Fig. 4.11 C).

In combination with pilocarpine, tropicamide showed a small and significant inhibition of pilocarpine-induced saliva secretion in rats when compared to vehicle-treated animals (Fig. 4.11 A & B). The ID_{50} was 13.6 mg/kg (95 % CI = 8.2 – 22.7 mg/kg).

Central administration of tropicamide (3.5 – 211 nmol i.c.v., equivalent to 1 and 60 µg) into the lateral ventricle had no effect on basal reduction of salivation (Fig. 4.12 C).

Tropicamide had no significant effect on pilocarpine-induced saliva secretion (Fig. 4.12 A and B). The ID_{50} could not be determined.

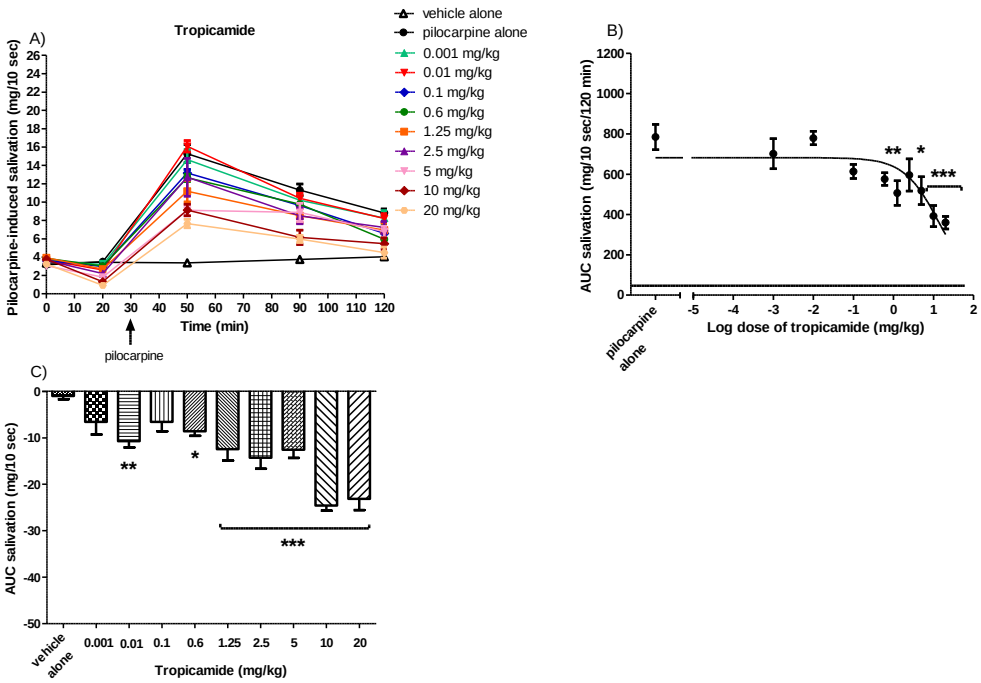


Figure 4.11 Effect of tropicamide (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 7). B) Log-dose response for tropicamide (0.001 – 20 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} = 13.6 mg/kg ± 1.3 mg/kg; (95 % CI = 8.2 – 22.7 mg/kg; r^2 = 0.37, $p<0.05$); *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared to vehicle-treated animals (One way ANOVA ($p<0.001$; F = 6.744; Df = 9, 60) followed by Dunnett's Multiple Comparison test). C) Effect of tropicamide on saliva within the first 20 min (AUC negative peaks time course). *$p<0.05$; **$p<0.01$; ***$p<0.001$ (One way ANOVA ($p<0.001$; F = 14.55; Df = 9, 60) followed by Dunnett's Multiple Comparison test).

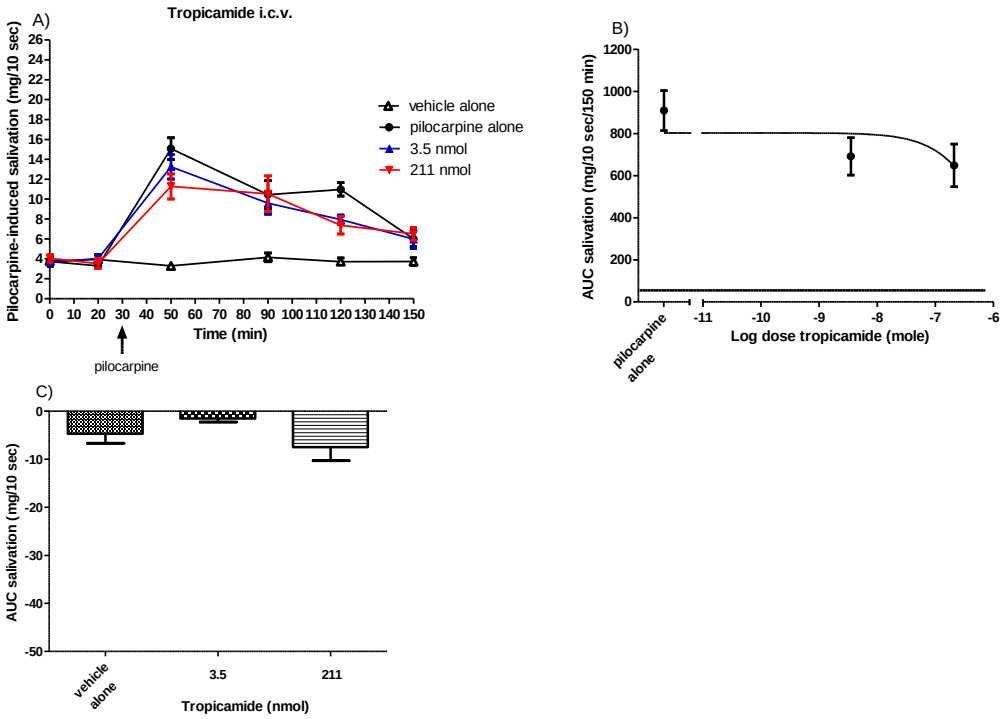


Figure 4.12 Effect of tropicamide (i.c.v.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for tropicamide (3.5 – 211 nmol i.c.v., equivalent to 1 & 60 µg) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} > 211 nmol; (r^2 = 0.07, NS); NS compared to vehicle-treated animals (One way ANOVA (p=0.1418; F = 2.147; Df = 2, 21). C) Effect of tropicamide on saliva within the first 20 min (AUC negative peaks time course). NS (One way ANOVA (p=0.1414; F = 2.151; Df = 2, 21)).

The novel M4 selective antagonist NBI-675 (0.1 – 30 mg/kg i.p.) showed no effect on basal salivation within 50 min when administered alone (Fig. 4.13 C).

NBI-675 did not alter pilocarpine-induced saliva secretion at lower doses, however, unexpectedly at the highest dose increased pilocarpine-induced salivation was observed, which was significantly different when compared to vehicle-treated animals (Fig. 4.13 A & B).

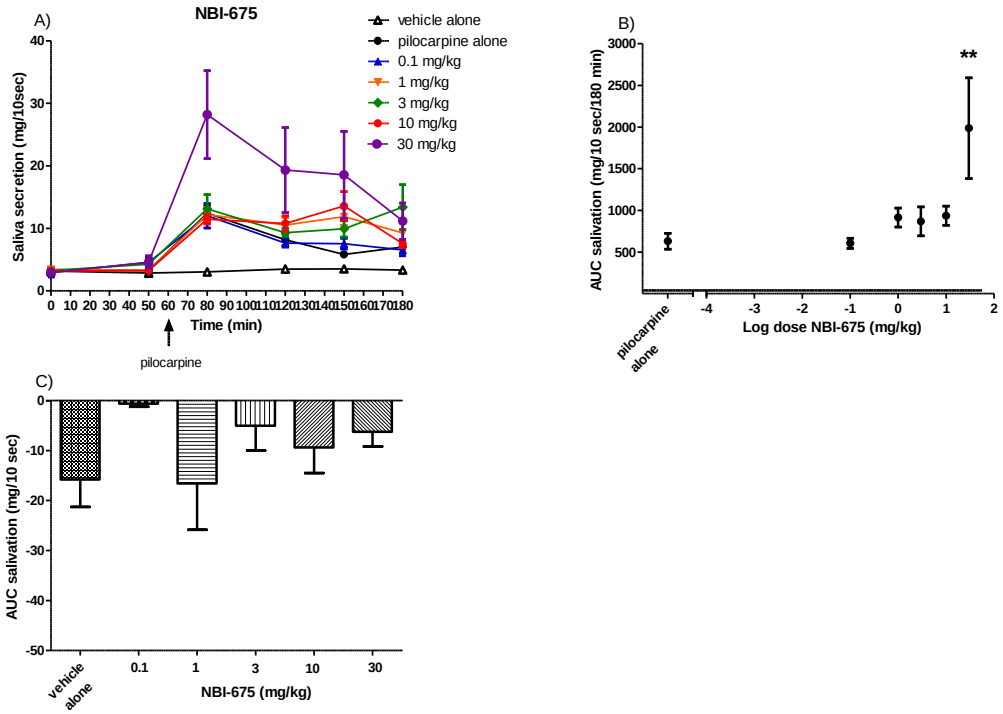


Figure 4.13 Effect of NBI-675 (i.p.) on pilocarpine-induced saliva secretion.
A) Time course. Data are mean ± SEM (n = 8). B) Log-dose response for NBI-675 (0.1 – 30 mg/kg i.p.) on suppression of pilocarpine-induced (3.4 mg/kg i.p.) saliva secretion (AUC time course). Data were analysed by a non-linear curve fit; the bottom was constrained to the mean of vehicle alone values (black line). ID_{50} > 30 mg/kg; (data not converged) **p<0.01; compared to vehicle-treated animals (One way ANOVA (p<0.01; F = 3.583; Df = 5, 42) followed by Dunnett's Multiple Comparison test). C) Effect of NBI-675 on saliva within the first 50 min (AUC negative peaks time course). NS (One way ANOVA (p=0.2663; F = 1.340; Df = 5, 42)).

Table 4.1 Summary of the inhibitory effect of anticholinergics on pilocarpine-induced saliva secretion.
- no effect; + small inhibition; ++ moderate inhibition; +++ high inhibition.

Anticholinergics	Subtype	Effect
Scopolamine i.p.	Non-selective	+++
Methylscopolamine i.p.	Non-selective	+++
Trihexyphenidyl i.p.	M1	+
Trihexyphenidyl i.c.v.	M1	++
Benztropine i.p.	M1	++
Pirenzepine i.p.	M1	+++
Pirenzepine i.c.v.	M1	-
AF-DX 116 i.p.	M2	+
Darifenacin i.p.	M3	+++
Darifenacin i.c.v.	M3	+
Tropicamide i.p.	M4	+
Tropicamide i.c.v.	M4	+
NBI-675 i.p.	M4	-

4.3.3 Other effects of anticholinergics treatment

As described previously in Section 3.3.3, administration of anticholinergics not only suppressed pilocarpine-induced salivation, but also reduced other peripheral and central effects of pilocarpine, including diarrhoea, urination, piloerection and purposeless chewing (Table 3.3), which were observed but not analysed quantitatively. Additionally, higher doses of centrally acting anticholinergics, particularly pirenzepine and NBI-675, produced CNS effects which were observed in animals and were manifested by reduced activity and movement, recumbency, and overall somnolence, which could be an indication of the CNS anticholinergics effects.

4.4. Discussion

Oral dryness (xerostomia) is a very common and unpleasant side effect of the anticholinergics treatment, reported in patients suffering from PD and dystonia, and is triggered by suppression of muscarinic receptors localised on salivary glands (Scully, 2003; Gautam *et al.*, 2004; Ekström *et al.*, 2012). Saliva secretion is regulated by both the sympathetic and parasympathetic branches of the autonomic nervous system. Adrenergic receptors of the sympathetic branches of the nervous system are responsible for content of proteins in saliva, whereas, muscarinic receptors of the parasympathetic nervous system responsible for volume of saliva secretion (Grisius, 2001).

In light of the results from previous study (Chapter 3) and current knowledge about side effect of anticholinergic treatment, it was hypothesised that muscarinic M4 receptor antagonists will reduce dystonia without induction of unfavourable side effects, such as dry mouth. The present study set out to investigate and compare the effect of anticholinergics with different selectivity for muscarinic receptor, with a special emphasis on the novel M4 selective muscarinic antagonists, NBI-675, on pilocarpine-induced saliva secretion in rats as an index of propensity to induce dry mouth.

Pilocarpine has been used in several preclinical and clinical studies in the assessment of salivary dysfunction and to confirm the involvement of muscarinic receptor subtype in mediation of saliva secretion (Ferguson, 1993; Wiseman & Faulds, 1995; Fox *et al.*, 2001; Sato *et al.*, 2006). As shown in the current and previous studies peripheral administration of pilocarpine produced excessive saliva secretion, which was visible within few minutes of administration (Chapter 2) (Gautam *et al.*, 2004; Sato *et al.*, 2006). Pre-treatment with antagonists and their effect on pilocarpine-induced salivation produced differential results and will be discussed below.

4.4.1 Which muscarinic receptor subtypes are responsible for salivation?

One of the aims of the study was to confirm muscarinic receptor subtype responsible for mediation of salivation and to understand the role of M4 component in this effect. Assessment of various anticholinergics on pilocarpine-induced saliva secretion confirms involvement of M3 receptors in mediation of salivation, since systemically administered darifenacin, a highly selective M3 antagonist, strongly abolished

salivation induced by pilocarpine. Previous studies have demonstrated that salivation is mediated by muscarinic M3 receptors located in salivary glands, thus these results are in agreement with earlier published research (Baum, 1993; Nakamura *et al.*, 2004; Langmead *et al.*, 2008). In addition, as the same doses of the compound evoked strong inhibition of salivation along with the moderate suppression of purposeless chewing, as described in previous chapter, this could suggest that M3 receptors are not ideal target for the treatment of dystonia. Interestingly, the selective M1 antagonist, pirenzepine, completely suppressed pilocarpine-induced saliva secretion when administered systemically. These results concur with the previous proposition that activation of both subtypes of receptors contribute to the production of saliva, as M1 receptor subtypes are also present in glands and are thought to be co-localised with M3 receptors (Culp *et al.*, 1991; Tobin, 1995; Tobin *et al.*, 2002; Eglen, 2006; Proctor & Carpenter, 2007). In addition, since pirenzepine does not cross the BBB, systemic administration of the drug resulted in blockade of the peripheral effects of pilocarpine, such as defecation, urination, piloerection and lachrymation. However, whilst peripheral administration of pirenzepine almost completely abolished pilocarpine-induced salivation, the relatively M1 selective antagonists, trihexyphenidyl and benztropine, produced more variable effect. This differential effect on salivation may be the consequence of antagonist selectivity for the different muscarinic receptors. Pirenzepine is considered to be highly selective for the M1 receptor, whereas both trihexyphenidyl and benztropine, although considered to be M1 selective, in fact bind with moderate to high affinity to all muscarinic receptors. Although both trihexyphenidyl and benztropine are used clinically in the treatment of dystonia and PD (Adam & Jankovic, 2007; Cloud & Jinnah, 2010) dry mouth may be a problem in some patients, even though the lack of dose-relationship suggests that this might be unpredictable.

The results obtained for AF-DX 116 indicate that M2 receptors are less likely to be involved in salivation, as the compound had no effect on reduction of salivation on its own or in combination with pilocarpine.

On the other hand, tropicamide, a relatively selective M4 antagonist, reduced saliva levels within 20 minutes of administration and produced a small suppression of saliva secretion induced by pilocarpine. By contrast, the highly selective M4 antagonist, NBI-675 showed no effect on inhibition of salivation on its own, and did not abolish

pilocarpine-induced salivation. However, very surprisingly and unexpectedly, the highest dose manifested in a notable and significant increase in salivation. The reason for this effect is unclear, but it could be related to the loss of the compound selectivity at the highest dose. However, since the lower doses suppressed pilocarpine-induces chewing (Chapter 3) but had no effect of salivation, these finding also suggest a beneficial effect of targeting M4 receptors in the prevention of dystonia without producing dry mouth, although the use of a more selective compound may be required.

It is important to note that studies on muscarinic M1/M3 double-KO mice showed that pilocarpine was unable to stimulate saliva secretion when compared to their WT controls, whereas the levels of salivary flow was similar in other muscarinic subtypes KO mice (M1/M4, M2/M3, M2/M4) when compared to their WT controls. This clearly demonstrates a role for M1 and M3 receptors in mediation of salivation (Gautam *et al.*, 2004).

This was confirmed by Borella *et al.,* (2008) who showed that central administration of M1 (pirenzepine), M2/M4 (methoctramine) and M4 (tropicamide) selective anticholinergics, had no effect on suppression of pilocarpine-induced salivation (Borella *et al.*, 2008).

Consequently, the results of the current study agree with previously published data (Gautam *et al.,* 2004; Borella *et al.,* 2008) and indicate that activation of muscarinic M1 and M3 receptors stimulate salivary flow, but that M2 have no effect and M4 could have little contribution to the saliva secretion.

4.4.2 Is pilocarpine-induced salivation mediated via central or peripheral muscarinic receptor?

Both scopolamine and its peripherally acting derivative, methylscopolamine, significantly reduced basal salivation levels. In addition, salivation in response to pilocarpine stimulation was completely and dose-dependently inhibited by both of the compounds. Although, they are classed as non-subtype specific, their *in vitro* pKi (selectivity) values are comparable to all muscarinic subtypes, and they show very similar values for both M1 and M3 receptors (Lazareno *et al.*, 1990; Caulfield & Birdsall, 1998) (Chapter 1, Table 1.5). A similar strong inhibitory effect of scopolamine was seen on purposeless chewing induced by pilocarpine, indicating both

central and peripheral properties of scopolamine, while peripheral administration of methylscopolamine lacked this central effect, as was unable to suppress pilocarpine-induced purposeless chewing (Chapter 3).

In contrast to the antagonistic effect of peripherally applied anticholinergics, none of the centrally administered compounds tested were able to inhibit salivation stimulated by pilocarpine. Neither i.c.v. administration of the M3 selective antagonist, darifenacin, nor the relatively selective M1 antagonist, trihexyphenidyl, were able to antagonise pilocarpine-induced salivation, confirming that the muscarinic control of salivation is peripherally mediated (Culp *et al.*, 1991; Gautam *et al.*, 2004). Likewise, i.c.v. administration of tropicamide and pirenzepine showed no effect on pilocarpine-induced salivation, confirming the lack of involvement of central M1 receptor in this response. Importantly, these results agree with previous work, which has demonstrated no inhibitory effect of central administration of pirenzepine and tropicamide on pilocarpine-induced salivation (Borella *et al.*, 2008). Although, i.c.v. tropicamide showed no effect on pilocarpine-induced salivation and purposeless chewing, this could be related to the poor solubility of the compound, which did not allow further testing. However, similar doses of pirenzepine dose-relatively supressed pilocarpine-induced purposeless chewing with a complete inhibition at the highest dose. Thus, the likely conclusion is that both tropicamide and pirenzepine were tested at active doses and that neither were able to inhibit saliva secretion.

Despite the fact that the lowest doses of pirenzepine did not alter the increased saliva levels induced by pilocarpine, higher doses of the drug produced marked and significant increase in salivation. Unexpectedly, this effect was also seen after systemic administration of the highest doses of NBI-675. This was perhaps unpredicted and surprising considering the fact that salivation is induced by activation of muscarinic receptors, rather than inhibition. The reason for this increase is unclear, however, since it was only observed at the highest doses of the compounds, it may be related to off target effects, loss of selectivity at higher doses, or central side effect of the anticholinergics treatment, such as confusion, drowsiness or sedation (Cloud & Jinnah, 2010; Wawruch *et al.*, 2012; Lampela *et al.*, 2015) which could have triggered behavioural changes observed during the assessment. Animals receiving high doses of these drugs not only exhibited the reduction in pilocarpine-induced purposeless

chewing, but also appeared to be somnolent, with overall reduced movement and mostly recumbent, which could possibly be due to the suppression of CNS activity. This could have reduced swallowing and built up of saliva in the mouth between the measurements, without actually increasing saliva secretion. Further studies investigation salivation with other sedative agents are required to confirm this supposition.

In many cases during the study pilocarpine-induced saliva secretion tended to be inhibited within the first assessment points, and then showing a peak at about 90 min after pilocarpine administration. The reason of this discrepancy is unclear, but the possibilities could include the duration of drug action, study design or different methods of measure the salivation. In previous studies saliva levels were measured in anaesthetised animals by placing cotton balls into their mouth (Renzi *et al.*, 2002; Borella *et al.*, 2008) or by collecting saliva into the sample tubes (Tobin *et al.*, 2002; Nakamura *et al.*, 2004; Sato *et al.*, 2006), whereas the current study was designed to obtain a salivation dose response of the effect of the drug in non-anaesthetised animals. In addition, normal physiological activities of animals, such as chewing, swallowing, grooming, could have had an impact on variability of obtained results. Although these could have not been prevented in this study, it was assumed that they were the same in all animals.

In summary, the present study indicate that peripherally located muscarinic receptors, in particular M1 and M3 are responsible for pilocarpine-induced salivation and indeed these results agree with previous work (Tobin *et al.*, 2002; Gautam *et al.*, 2004; Borella *et al.*, 2008). Importantly, the results confirm previous suggestion that muscarinic M4 receptors do not play a major role in mediation of saliva secretion, as highly selective NBI-675 failed to inhibit pilocarpine-induced salivation.

4.5. Conclusion

The current study aimed to compare the ability of various anticholinergics to inhibit pilocarpine-induced saliva secretion in rats and confirm the receptor subtype responsible for mediation of this process. Results confirm previous findings that muscarinic M1 and M3 receptors are responsible for promoting salivation (Tobin *et al.,* 2002; Gautam *et al.,* 2004; Borella *et al.,* 2008). Importantly, this investigation also shows that muscarinic M4 receptors do not play a major role in mediation of salivation, since the selective antagonist NBI-675 failed to suppress pilocarpine-induced saliva secretion in rats at doses that were at the lower end of the DRC that inhibited chewing. As a consequence, the hypothesis of this study that selective antagonism of muscarinic M4 receptors which reduce dystonia will not induce unfavourable oral dryness can be accepted. Importantly, studies in the previous and current chapter show that selective antagonism of muscarinic M4 receptors suppressed involuntary movements without induction of unfavourable peripheral side effects, such as dry mouth, therefore muscarinic M4 receptors could be a target for treatment of dystonia. However, it would be important to test drug effects in other models of dystonia. One of the well-established model to investigate drug-induced involuntary movements is a non-human primate model, the MPTP-treated marmosets, primed to express permanent 'on-drug' dystonia and chorea (dyskinesia) following chronic L-DOPA treatment. Therefore, the investigation described in subsequent chapter will focus on examination of the effect of anticholinergics of different selectivity in the MPTP-treated marmosets model of Parkinson's disease to assess whether selective antagonism of muscarinic M4 receptors have the potential to improve motor disability.

Chapter 5 The effect of anticholinergics on parkinsonian disability in MPTP-treated common marmosets

5.1. Introduction

In the previous chapters, it was reported that selective antagonism of central muscarinic M4 receptors can suppress involuntary movements with reduced unfavourable peripheral side effects seen with non-selective antagonism in rats. From this it was concluded that directly targeting muscarinic M4 receptors may show promise for the treatment of dystonia where non-selective antagonists are effective, but compliance is poor. Anticholinergics are also used to treat other diseases, including the motor symptoms of Parkinson's disease (PD), and this raises the question as to whether M4 antagonists may have a similar beneficial effect in this disease. However, the studies described in Chapters 3 and 4 used a pharmacological model of dystonia, they do not fully interrogate the potential use of selective muscarinic antagonists in the modulation of motor dysfunction in PD, where striatal cholinergic function is altered due to the loss of afferent neuronal control of cholinergic interneurons (section 1.2.1.2).

As described earlier (section 1.1.2) PD is the most common hypokinetic movement disorder where the loss of DA input into the striatum triggers underactivity of the thalamocortical pathway, and reduction in movement, mediated by overactivity in the indirect and underactivity in the direct striatal output pathways in the basal ganglia (section 1.2.1.1). This imbalance between the two basal ganglia circuits results in motor dysfunction, including bradykinesia, rigidity, tremor and postural instability, the cardinal symptoms of PD (Bonsi *et al.*, 2011; Benarroch, 2012). The reduced dopaminergic activity in the striatum results in increased release of acetylcholine from striatal interneurons, explaining the beneficial use of anticholinergics in the treatment of PD (Duvoisin, 1967; Schapira, 2005). Originally this increase in ACh was thought to be related solely to the disinhibition of D2 receptor activation on ChI (Maurice *et al.*, 2004). However, it is now understood that muscarinic receptors play an important role in the control of striatal dopaminergic afferents and GABAergic efferents. In particular, M4 receptors are expressed postsynaptically on D1 MSN and may play a vital inhibitory role in the activity of the direct pathway (Gomeza *et al.*, 1999a; Onali & Olianas, 2002; Pisani *et al.*, 2007), thus contributing to the DA-ACh balance in the activity of D1 MSN (Onali & Olianas, 2002).

A number of preclinical studies have investigated the effect of targeting muscarinic receptors, in particular M4 subtype, in the treatment of PD. Rodent studies have shown that tropicamide, which is only relatively selective for M4 receptors, can reverse parkinsonian symptoms induced by administration of systemic non-selective muscarinic agonist pilocarpine (Betz *et al.*, 2007), a model similar to that described in Chapter 3 of this thesis. The authors suggestion that one would expect fewer side effects due to the high expression of the M4 receptors within the striatum and lower abundance in the periphery compared to other subtypes of muscarinic receptors (Betz *et al.*, 2007; Langmead *et al.*, 2008) was confirmed in the studies described in this thesis (Chapter 4) with the highly selective M4 antagonist, NBI-675.

Moreover, Karasawa et al., (2003) reported that haloperidol-induced catalepsy was inhibited by scopolamine in wild-type mice, whereas this anticholinergic was unsuccessful in blocking cataleptic responses in M4 knockout (KO) mice, indicating involvement of M4 receptor in motor control (Karasawa *et al.*, 2003).

Nevertheless, MPTP-treated primate model is by far the best model of PD to study motor symptoms seen in man. The MPTP-treated common marmosets display dopaminergic loss in the substantia nigra and loss of striatal dopaminergic terminals accounting for 90 – 95% (Jenner *et al.*, 1984). This model mimics mid to late stage PD, and as such provides an effective tool to study novel treatment of the motor symptoms of the disease. Measures of motor function, including locomotor activity and motor disability are used to provide the insights into the drug treatment. In addition, chronic dopaminergic treatment results in the expression of dyskinesia which mimic those seen after long-term treatment in PD (Kuoppamaki *et al.*, 2007).
Although anticholinergics are effective in the treatment of PD, as with the treatment of dystonia, patients experience similar unpleasant peripheral side effects. There is a limited number of studies investigating the effects of anticholinergics in models of PD. However, in MPTP-treated primates it has been reported that non-selective centrally acting anticholinergics dose-dependently reversed motor disability and reduced akinesia (Jackson *et al.*, 2014), but this was accompanied by side effects, including mydriasis, incoordination and difficulty in eating (Close *et al.*, 1990).

Overall, these observations indicate the muscarinic involvement in motor control and suggest that muscarinic antagonists, and particularly those selective for the M4

receptor, are candidates in the relieving the motor symptoms of PD. However, no studies to date have investigated the effect of the selective M4 antagonists in the MPTP-treated primates due to the lack of the appropriate compounds. As reported in the previous chapter, the selective M4 antagonist, NBI-674, showed central anticholinergic activity, blocking pilocarpine-induced perioral movements with reduced peripheral side effects compared to less selective muscarinic receptor antagonists, allowing the investigation of the effects of selective M4 antagonists in the treatment of the motor symptoms of PD for the first time.

5.1.1. Hypothesis

It is hypothesised that selective antagonism of muscarinic M4 receptors with NBI-675 will relieve motor symptoms of the MPTP-treated marmoset model of Parkinson's disease, when given alone and will enhance the antiparkinsonian effects of L-DOPA, when given in combination.

5.1.2. Aims

In order to test this hypothesis these studies aimed to compare the effect of selective inhibition of M4 receptors with other non-selective or non-M4-selective antimuscarinics, when given alone and in combination with L-DOPA, on the reversal of motor deficits in the MPTP primate model of Parkinson's disease. The specific aims of this study were to:

1) Confirm that L-DOPA reverses motor symptoms in MPTP-treated common marmosets.
2) Determine the role of anticholinergics of different selectivity to muscarinic receptors on the reversal of motor symptoms in MPTP-treated common marmosets.
3) Determine whether anticholinergics alter L-DOPA-induced reversal of motor disability.
4) Determine whether the anticholinergic NBI-675 alters the pharmacokinetics of L-DOPA by using HPLC-ECD.

5.2 Materials and Methods

In order to address these aims the following studies were performed:

1) The effect of L-DOPA on locomotor activity and reversal of motor disability was investigated by treating MPTP-treated common marmosets by L-DOPA or vehicle. Drug effect was determined by changes in locomotor activity and motor disability.
2) The role of clinically used anticholinergics alone and their effect on L-DOPA increase of locomotor activity and reversal of motor disability was investigated by treating MPTP-treated common marmosets with peripheral and central acting anticholinergics 1 hr prior to L-DOPA or vehicle. Changes in locomotor activity and motor disability were assessed to determine drug effect.
3) The role of the M4 muscarinic receptor in L-DOPA increase of locomotor activity and reversal of motor disability was investigated using the novel selective M4 antagonist NBI-675 administered to MPTP-treated common marmosets 1 hr prior to L-DOPA or vehicle. Changes in locomotor activity and motor disability were assessed to determine drug effect.
4) The effect of NBI-675 on L-DOPA pharmacokinetics was determined by measuring the levels of L-DOPA and its metabolite 3-OMD in blood plasma up to 3 hr after administration of L-DOPA. Plasma levels of L-DOPA and metabolites were measured by HPLC with electrochemical detector.

An overview of the methods used is described below with detailed methodology found in Chapter 2.

5.2.1 Animals

Adult common marmosets (*Callithrix Jacchus*) (Harlan, UK, 350 – 500 g, n = 6-8 per group) of either sex were used in this study. Animals were previously treated with MPTP and primed with L-DOPA and were not drug naïve prior to the study. For details please refer to Chapter 2 section 2.2.3.1. All experiments were carried out in accordance with Home Office regulations under the Animas (Scientific Procedures) Act 1986 and project licence number 70/7146 and 70/8541.

5.2.2 Behavioural assessment

As described in Chapter 2, all behavioural assessments were carried out between the hours 7.00 am and 3.00 pm. The assessment was performed for 1 hr before (baseline score) and 6 hr after anticholinergics/vehicle administration, as described in section 2.2.3.3 In brief, locomotor activity was measured by infrared beam interruptions. Animals were observed through one-way mirror by experienced observers blinded to the drug treatment through one-way mirror and scored for motor disability reversal (Chapter 2, Fig. 2.5) during the last 10 min of each consecutive 30 min intervals. Full description of assessment criteria are provided in sections 2.2.3.3.

Locomotor activity, motor disability and dyskinesia with dystonia and chorea, were assessed during one experiment on the same animals, however, for the clarity, behavioural outcomes were split into two chapters to assess the effects of anticholinergics on motor function (Chapter 5) and on L-DOPA-induced dyskinesia (Chapter 6).

5.2.3 Drug treatment

Full details of drug preparation and treatment are described in Chapter 2, section 2.2.3.4. Briefly, on test days animals were placed into the testing units and were given 60 min acclimatization in the test cages prior to drug treatment. Following the 60 min acclimatisation period which provided baseline activity data, animals were dosed with appropriate anticholinergic or vehicle (s.c. or p.o.) followed by L-DOPA (8 mg/kg p.o.) + benserazide (10 mg/kg p.o.) or vehicle 60 min later. Doses of anticholinergics are presented in Table 5.1. Behavioural assessment was then carried out as described in Chapter 2 section 2.2.3.3. A repeated crossover design was used, so each marmoset was treated with all doses of a single drug or vehicle with at least 72 hr washout between the tests. A typical modified Latin square was used to randomise drug treatments and is shown in Table 5.1.

Table 5.1 Typical example of a latin square with a crossover designed treatment for administration of different doses of anticholinergics prior L-DOPA.

Animal number	Treatment day					
	1	2	3	4	5	6
1	A	B	C	D	E	F
2	D	A	E	B	F	C
3	C	F	A	E	D	B
4	B	D	F	A	C	E
5	E	C	B	F	A	D
6	D	B	E	C	F	A
7	A	E	C	D	B	F
8	F	A	D	E	C	B

1) A = vehicle anticholinergic + L-DOPA
2) B = dose 1 anticholinergic + vehicle L-DOPA
3) C = dose 1 anticholinergic + L-DOPA
4) D = dose 2 anticholinergic + vehicle L-DOPA
5) E = dose 2 anticholinergic + L-DOPA
6) F = vehicle anticholinergic + vehicle L-DOPA

Locomotor activity was recorded throughout the study. Motor disability was scored immediately before each drug treatment and then every 30 minutes for 10 minute periods throughout the study for up to 5 hours, as shown in Chapter 2 Figure 2.7. Full description of assessment criteria are provided in sections 2.2.3.3.

One animal was taken out of the study due to seizures caused by the highest dose of NBI-675 on the last day of study.

Table 5.2 Doses and route of administration of drugs used in the study.

Drug	Dose	Route
Benztropine	0.25 & 0.5 mg/kg	s.c.
Scopolamine	0.1 & 0.3 mg/kg	s.c.
Methylscopolamine	0.1 & 0.3 mg/kg	s.c.
Trihexyphenidyl	0.5 & 1 mg/kg	p.o.
NBI-675	1, 5 & 7.5 mg/kg	p.o.
L-DOPA + benserazide	8 mg/kg p.o.+ 10 mg/kg	p.o.

5.2.4 Plasma exposure – blood sampling

To determine whether the NBI-675 alters the pharmacokinetics of L-DOPA, the levels of L-DOPA and its metabolite 3-OMD in blood plasma were assessed using HPLC with electrochemical detector.

Animals were placed in the automated activity (test) cages. They were allowed 60 min of acclimatization, and were dosed with NBI-675 (5 mg/kg p.o.) followed by L-DOPA (4 mg/kg p.o.) + benserazide (10 mg/kg p.o.) 1 hour later. No behavioural assessments were performed. At the hour 1 and 3 after L-DOPA treatment, animals were removed from the cage (one at the time), hand restrained, the inner side of the thigh was disinfected with hibiscrub diluted in water (1:10 hibiscrub to water), 0.4 ml of blood was withdrawn from the femoral vein using 25G needle. The blood sample was collected into the heparin tubes and kept on ice. A gauze with adequate finger pressure was applied immediately to the site until bleeding stopped. Animals received a reward, a marshmallow, and were returned into their cages. The alternate legs were used to obtain blood samples. After all samples were collected, they were centrifuged at 4˚C at 5000 rpm for 10 min. Supernatant (blood plasma) was collected into the Eppendorf tubes and frozen at -70˚C until further use.

5.2.5 HPLC-ECD detection of L-DOPA and its metabolites

Concentrations of L-DOPA and 3-O-methyldopa (3-OMD) were analysed in blood plasma samples of common marmosets by high pressure liquid chromatography with an electrochemical detector (HPLC-ECD).

5.2.5.1 Sample preparation

The methods of sample preparation were performed according to a modification of methods by Rose et al., (1988). To determine plasma concentration of L-DOPA and its metabolite, 3-OMD, blood plasma samples were thawed at 4˚C on ice and mixed (9:1 volume/volume ratio) in 0.4 M perchloric acid (PCA) solution containing 1 mM EDTA disodium and 0.01% sodium metabisulphate (referred to as PCA solution). The PCA solution also contained 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard at a final concentration of 1.0 µg/ml. To further acidify the samples 30% (volume/volume) of 70% (weight/volume) PCA solution was added. Plasma samples were then sonicated using a Microson ultrasonic tissue disruptor to progress

acidification of proteins. The samples were then centrifuged at 4˚C at 13000 rpm for 10 min and the supernatant was collected and analysed by HPLC-ECD.

5.2.5.2 HPLC-ECD

Standard solutions of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) were prepared in the PCA solution at a concentration of 1 mg/ml individually and stored at -70ºC. DHBA (1 mg/ml in PCA) was also prepared separately and stored at -70ºC. Stock solutions of L-DOPA (1 mg/ml) and 3-OMD (1 mg/ml) in PCA solution were prepared on the day of experiment. A range of standard concentrations (200 ng/ml – 5 µg/ml for L-DOPA; 10 µg/ml – 250 µg/ml for 3-OMD) containing DHBA (1 µg/ml) was prepared for calibration. Dopamine, DOPAC, 5-HT, 5-HIAA and HVA were added into the standard to clearly differentiate L-DOPA and 3-OMD peaks although their concentrations were not determined in the blood plasma samples.

The UltiMate 3000 HPLC system consisted of a LPG-3400A pump, a WPS-3000TSL autosampler, a Spherisorb ODS (2) 3 µm particle size HPLC column (SpheriClone 0.46 cm x 10 cm; Phenomenex, UK) and a temperature regulated column compartment (all from Dionex, UK). They were coupled to INTRO Electrochemical detector (Antec, UK) incorporating a VT-03 flow cell with a spacer of 50 µm thickness and KCl reference electrode (Aquilant Scientific (Presearch), UK) through the UCI-50 Universal Chromatography Interface (Dionex, UK). The mobile phase was made of 0.1 M sodium dihydrogen orthophosphate buffer containing 12% HPLC grade methanol, 1 mM disodium EDTA and 56 mM octane-1 sulfonic acid in 18MΩ water and adjusted pH with 3 M orthophosphoric acid to pH 3.2. The mobile phase was filtered through polyamide membrane filters (0.2 µm pore size; Sartorius Stedim Ltd., UK) and degassed during the analysis. Isocratic elution was made at a flow rate of 0.8 ml/min. The column was maintained at 30ºC in the column compartment, and samples and standards were stored at 9ºC in the autosampler. A potential of 0.72 V was maintained across the glassy carbon working electrode. The system was calibrated

using 1 µg/ml standard every three samples. All HPLC settings employed in this study are listed in Table 5.3.

Table 5.3 Chromatographic conditions.

Column temp	30°C
Samples temp	9°C
Voltage	0.72V
Flow rate	0.8 ml/min (isocratic)
Filter	0.5 s
Range	5-20 nA/V
Injection volume	10 µl
Run per sample	15 min
Standard volume	1 µg/ml for L-DOPA 50 µg/ml for 3-OMD
Mobile phase	0.1M NaH_2PO_4 1mM EDTA disodium 0.56mM octane-1-sulfonic acid sodium salt (4% w/v) 12% methanol (HPLC grade) pH 3.2 with 3M phosphoric acid

5.2.5.3 Determination of L-DOPA and 3-OMD concentrations

Peak height of the analytes and internal standards were measured by Chromeleon 6.8 Chromatography Data System (Dionex, UK). Concentrations of the samples were determined by the comparison of peak height ratio (PHR) between the internal standard peak and analytes using the following equation:

$$\text{amount in sample} = \frac{\text{PH (sample L-DOPA)} \times [\text{STD}] \times \text{PH (STD DHBA)}}{\text{PH (sample DHBA)} \times \text{PH (STD L-DOPA)}}$$

where: PH = peak height and STD = Standard.

Calibration curves were calculated using the peak height to DHBA ratio (PHR) (Fig. 5.1). Data were expressed as ng/ml. Typical chromatographs for standards, blood plasma containing DHBA alone and blood plasma at 1 hr after L-DOPA administration are presented in Figure 5.2. Standard mixture solution at the concentration of 1.0 µg/ml was repeatedly injected into the system ten times and stability of peak height ratio for

L-DOPA and 3-OMD was calculated as an intra assay coefficient of variance. Intra assay coefficient of variance was 0.43% and 0.31% for L-DOPA and 3-OMD, respectively (Fig. 5.3). The recovery rates were 105 ± 1.18% and 103 ± 0.43% for L-DOPA and 3-OMD, respectively.

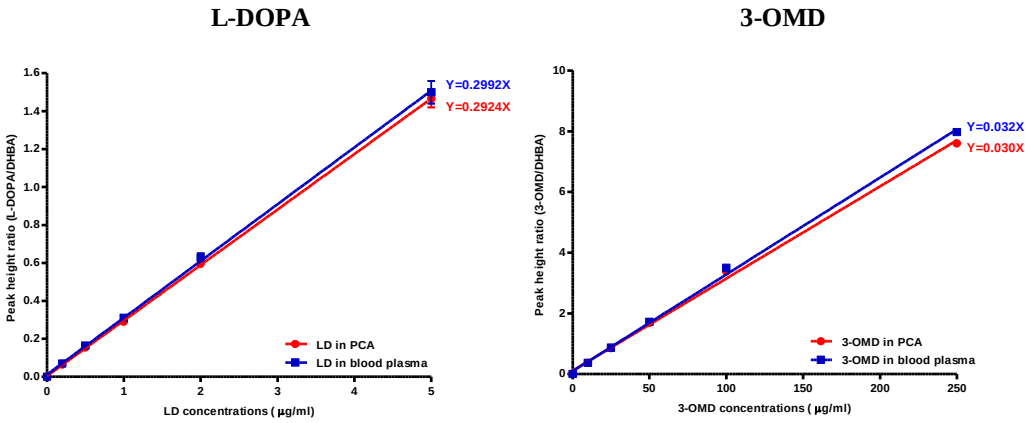


Figure 5.1 Representative calibration curves for L-DOPA and 3-OMD using the peak height ratio to DHBA in blood plasma.
Recovery rates were: L-DOPA = 105 ± 1.18%; 3-OMD = 103 ± 0.43%.

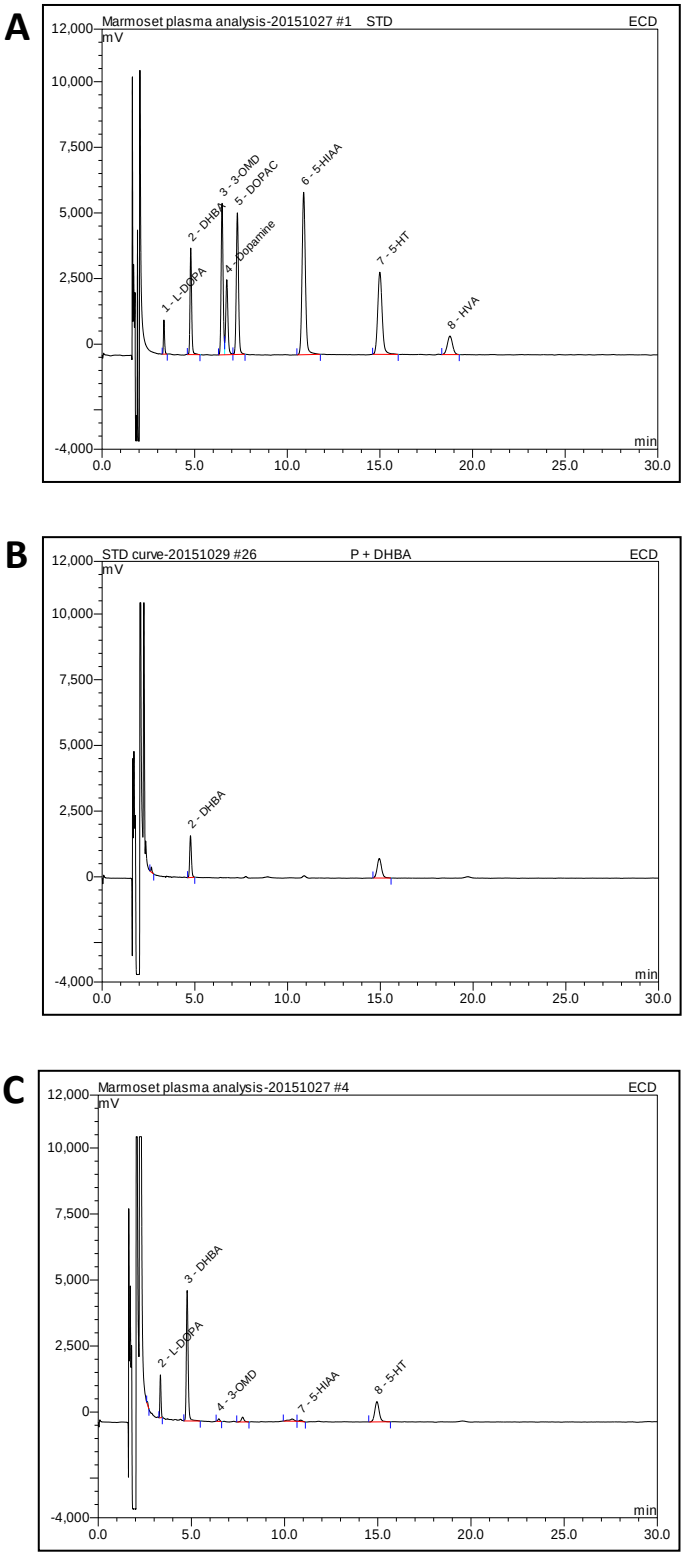


Figure 5.2 Representative chromatographs obtained by HPLC-ECD from common marmoset blood plasma.

A) standard; B) blood plasma containing DHBA alone and C) blood plasma at one hour after L-DOPA treatment.

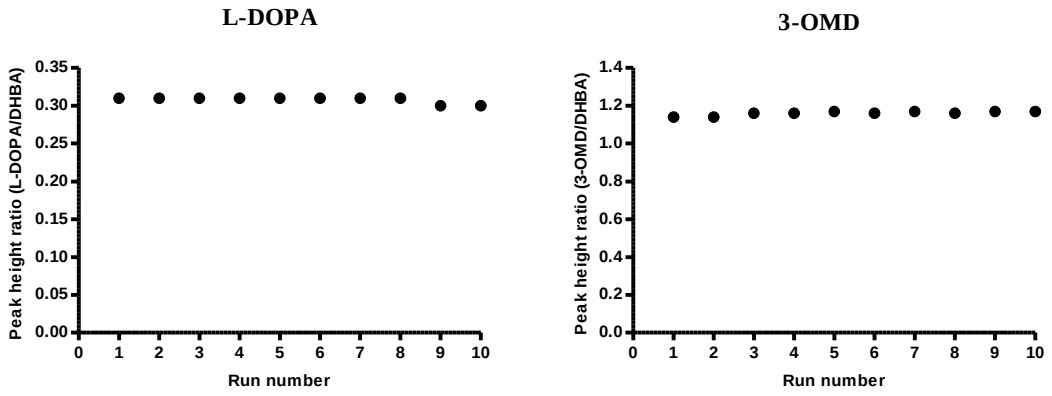


Figure 5.3 Typical intra assay stability of L-DOPA and 3-OMD over the repeated analysis for common marmoset blood plasma sample.
Stability of peak height ratio of L-DOPA and 3-OMD to DHBA was assessed as an intra assay coefficient of variation following repeated injections of standard solution (1.0 µg/ml). Intra assay coefficient of variation of the standards was 0.43% for L-DOPA and 0.31% for 3-OMD.

5.2.6 Statistical analysis

Data and statistical analysis were performed using GraphPad Prism 5.02 (San Diego, CA, USA) as described in Section 2.2.3.5, Chapter 2.

The statistical analysis for locomotor activity and motor disability data was performed as follows:

4) No statistical analysis was performed on time course data;
5) Totals (AUC_{-1-0h}) and (AUC_{0-5h}) and Peak for motor disability data were transformed by square root ($Y=\sqrt{Y}$).
6) Totals (AUC_{-1-0h}) and (AUC_{0-5h}), Peak, On-time, On-time > 2 were analysed by Two-way ANOVA (variables: L-DOPA and anticholinergic) and repeated measures ANOVA followed by *post hoc* Newman-Keuls multiple comparisons test.

Concentration of L-DOPA and 3-OMD in MPTP-treated common marmoset plasma following L-DOPA administration after 1 and 3 hours were determined using HPLC-ECD. Data are presented as mean ± SEM (n = 6) and were analysed by Two-way ANOVA (variables: time and treatment), if an effect was found then analysis was performed by Friedman's test followed by *post hoc* Dunn's test.

5.3 Results

In all studies vehicle-A-vehicle-B administration had no effect on locomotor activity or reversal of motor disability over time in MPTP-treated common marmosets (Fig. 5.4 – 5.13).

Following the acclimatisation time, and in the absence of L-DOPA, animals appeared akinetic and hunched, either sitting on the cage floor or on the perches. They were often staring in one direction, and at times looked sleepy, however, occasional alertness with head checking and overall slow movements were also present. Their locomotor activity scores were low with little variability (with a median t=-1-0 LMA score of 150.5, range 0 – 3428) reflecting the akinesia, and their motor disability scores were high (with a median t=-1-0 motor disability score of 11, range 2 – 14), as expected for a parkinsonian marmoset.

Administration of L-DOPA alone resulted in an increase in locomotor activity with a total duration of about 3.5 hrs and a peak effect between 30 min to 1 hr after administration before gradually returning to the baseline. This resulted in a significant increase in locomotor activity as measured by total counts ($AUC_{0\text{-}5h}$) (Fig. 5.6 B, 5.8 B & 5.10 B), on-time (Fig. 5.6 C, 5.8 C & 5.10 C) and peak activity (Fig. 5.4 D, 5.6 D, 5.8 D, 5.10 D & 5.12 D) when compared to vehicle-A-vehicle-B-treated animals.

Similarly, L-DOPA alone reversed motor disability with a total duration of around 3.5 hrs and a peak effect observed between 1 and 2.5 hrs after administration, before gradually returning towards baseline. This resulted in a significant increase in total motor disability score ($AUC_{0\text{-}5h}$) (Fig. 5.5 B, 5.7 B, 5.11 B & 5.3 B), increase in on-time (Fig. 5.5 C, 5.7 C, 5.9, 5.11 & 5.13 C) and reduction in peak score (Fig. 5.5 D, 5.7 D, 5.9 D & 5.11 D) when compared to vehicle-A-vehicle-B-treated animals.

Combination of L-DOPA and anticholinergics is described below for every compound separately, however, generally centrally acting anticholinergics appeared to extend the duration of L-DOPA effect.

5.3.1 The effect of non-selective anticholinergics

5.3.1.1 Centrally acting scopolamine

5.3.1.1.1 Locomotor activity

Scopolamine alone (0.1 & 0.3 mg/kg) produced an immediate increase in locomotor activity, which lasted for about 2 hrs and was not dose-dependent, resulting in a significant increase in locomotor activity counts over the first hour (AUC_{-1-0h}) prior to L-DOPA administration compared to vehicle-A (Fig. 5.4 E).

Pre-treatment with scopolamine (0.1 & 0.3 mg/kg) had no significant effect on L-DOPA-induced rise in locomotor activity, as measured by total counts (AUC_{0-5h}), on-time or peak activity when compared to L-DOPA alone (Fig. 5.4 B – D). When compared to scopolamine alone, the combination treatment remained significantly increased as measured by total counts (AUC_{0-5h}), on-time and peak activity (Fig. 5.4 B – D).

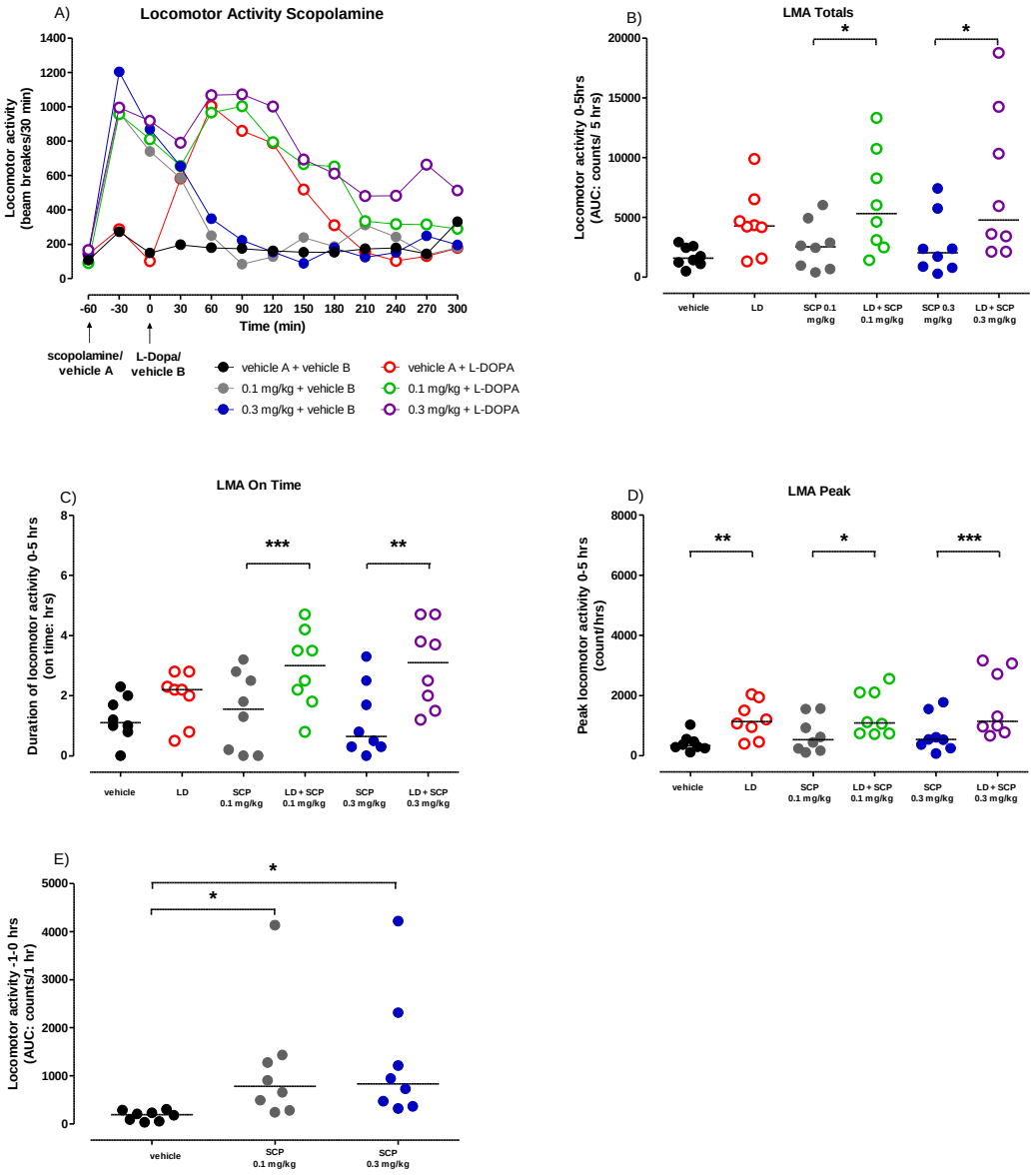


Figure 5.4 Effect of scopolamine on L-DOPA-induced locomotor activity in MPTP-treated common marmosets

Scopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Locomotor activity time course and B) Total locomotor activity ($AUC_{0\text{-}5h}$), C) On-time and D) Peak activity after L-DOPA/vehicle-B administration; E) Total counts in the first hour after scopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with mean values with error bars omitted for clarity (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; (B) F=6.806; Df (5,47); p=0.0002; C) F=8.310; Df (5,47); p<0.0001; D) F=10.23; Df (5,47); p<0.0001; E) F=5.284; Df (2,23); p=0.0195) followed by Newman-Keuls Multiple Comparison post hoc test *p<0.05; **p<0.01; ***p<0.001.

5.3.1.1.2 Motor disability

Scopolamine alone (0.1 & 0.3 mg/kg) produced an immediate reversal of motor disability with a peak effect at 30 min (Fig. 5.5 A). This effect was significantly greater than vehicle-A over the first hour (AUC_{-1-0h}) (Fig. 5.5 E) and resulted in a significant improvement in motor disability over the 5 hours after vehicle-B administration as measured by on-time and peak scores when compared to vehicle-A/B-treated animals (Fig. 5.5 C & D), although there was no effect of scopolamine on total scores (AUC_{0-5h}) when compared to vehicle-A/B-treated animals (Fig. 5.5 B).

There was no significant effect of scopolamine (0.1 & 0.3 mg/kg) on L-DOPA-induced reversal of motor disability as measured by total (AUC_{0-5h}) and peak scores and on-time when compared to L-DOPA alone (Fig. 5.5 B – D), although on-time tended to be increased.

Reversal of the motor disability by the L-DOPA/scopolamine combination was not significantly different to scopolamine alone (Fig. 5.5 B – D).

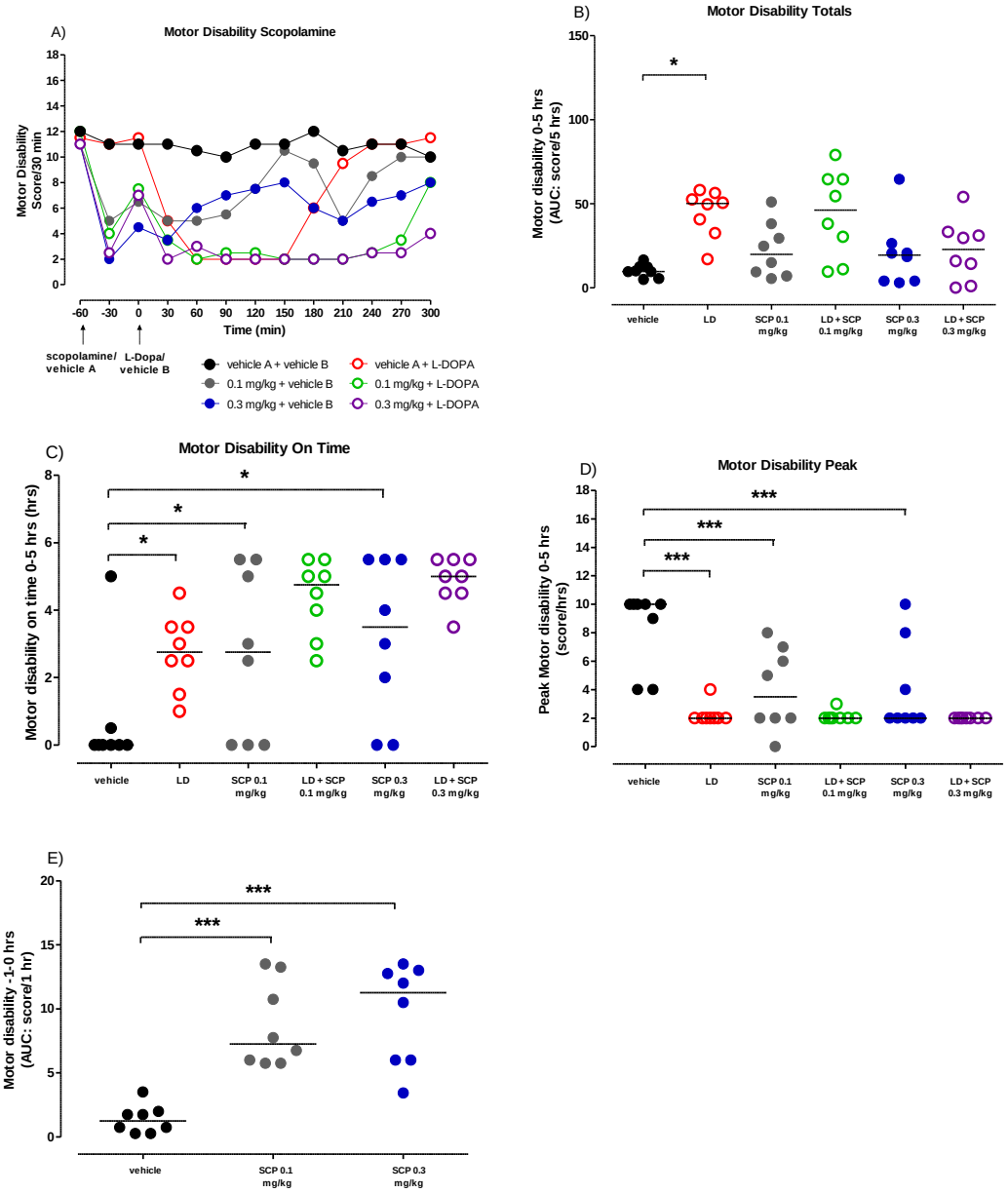


Figure 5.5 Effect of scopolamine on L-DOPA-induced reversal of motor disability in MPTP-treated common marmosets

Scopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Motor disability time course and B) Total motor disability reversal ($AUC_{0\text{-}5h}$) C) On-time and D) Peak motor disability after L-DOPA/vehicle-B administration; E) Total motor disability reversal in the first hour after scopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; For B, D & E data were transformed $y=\sqrt{y}$; (B) F=4.064; Df (5,47); p=0.0052; C) F=7.522; Df (5,47); p<0.0001; D) F=9.258; Df (5,47); p<0.0001; E) F=30.59; Df (2, 23); p<0.0001) followed by Newman-Keuls post hoc test *p<0.05; ***p<0.001.

5.3.1.2 Peripherally acting methylscopolamine

5.3.1.2.1 Locomotor activity

Methylscopolamine had no significant effect on locomotor activity as measured by total counts (AUC_{-1-0h}) and (AUC_{0-5h}), peak activity and on-time when compared to the vehicle-A/B treatments (Fig. 5.10 B – E).

Pre-treatment with methylscopolamine (0.1 & 0.3 mg/kg) had no effect on L-DOPA-induced locomotor activity when compared to L-DOPA alone (Fig. 5.6 B – D). When compared to methylscopolamine alone, the combination treatment L-DOPA/methylscopolamine remained significantly improved as measured by on-time only (0.3 mg/kg), while there was no increase in locomotor activity when measured by total counts (AUC_{0-5h}) and peak activity (Fig. 5.6 B – D).

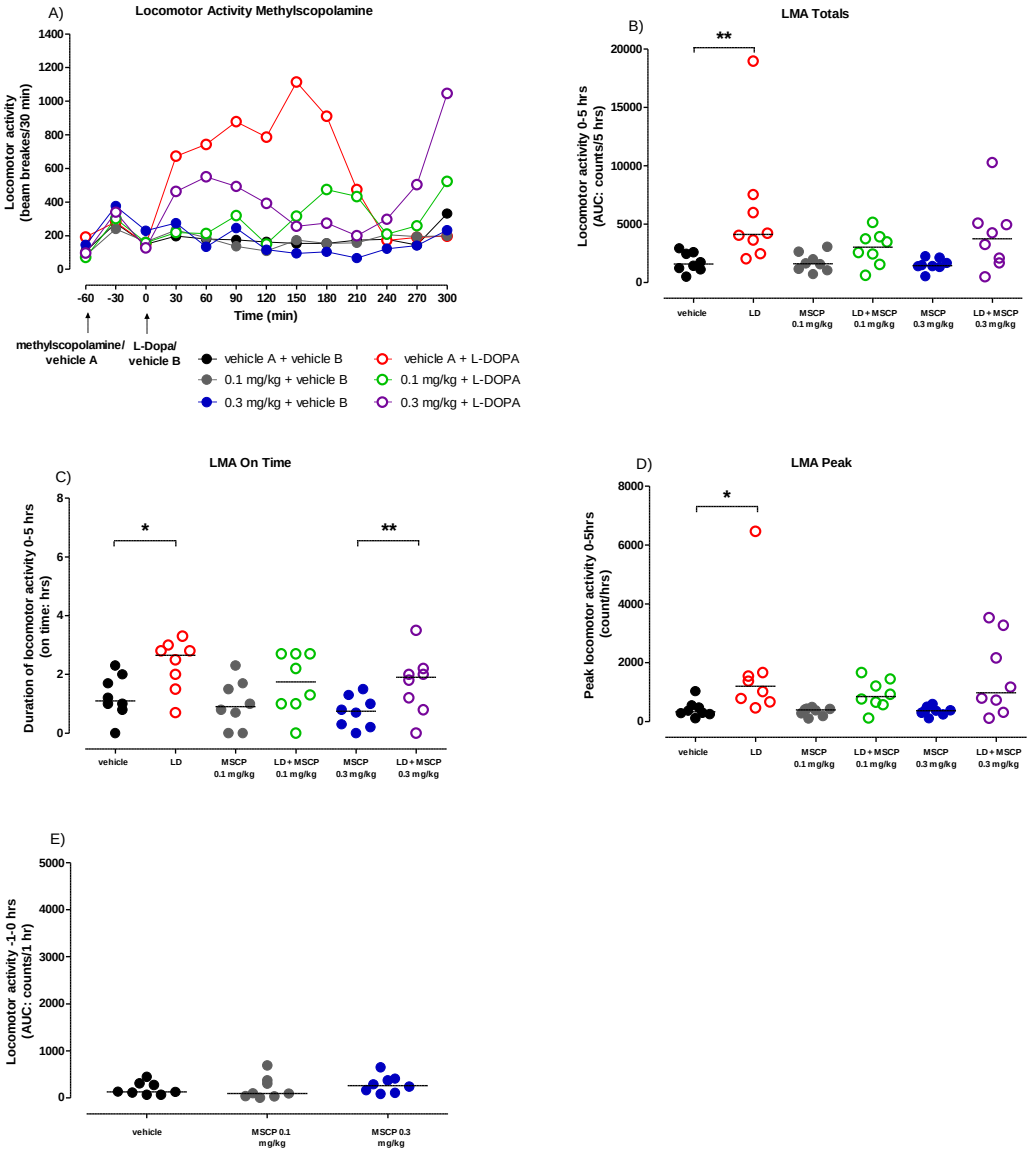


Figure 5.6 Effect of methylscopolamine on L-DOPA-induced locomotor activity in MPTP-treated common marmosets

Methylscopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Locomotor activity time course and B) Total locomotor activity ($AUC_{0\text{-}5h}$), C) On-time and D) Peak activity after L-DOPA/vehicle-B administration; E) Total counts in the first hour after methylscopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with mean values with error bars omitted for clarity (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; (B) F=3.824; Df (5,47); p=0.0073; C) F=6.904; Df (5,47); p=0.0001; D) F=3.817; Df (5,47); p=0.0073; E) F=1.206; Df (2,23); p=0.3288) followed by Newman-Keuls post hoc test *p<0.05; **p<0.01.

5.3.1.2.2 Motor disability

Methylscopolamine alone produced a very small but significant reversal of motor disability at the higher dose (0.3 mg/kg) when compared to vehicle-A treatment over the first hour (AUC_{-1-0h}) (Fig. 5.7 E), although the animals were not considered "on" as determined by a motor disability scores < 8. There was no overall effect of methylscopolamine after L-DOPA/vehicle-B administration when compared to vehicle-A/B (Fig. 5.7 B – D).

Pre-treatment with methylscopolamine significantly decreased L-DOPA-induced reversal of motor disability as measured by total (AUC_{0-5h}) scores (Fig. 5.7 B) and tended to reduce the duration of reversal of motor deficits as measured by on time when compared to L-DOPA alone (Fig. 5.7 C), although this was not significant. When compared to methylscopolamine alone (0.1 & 0.3 mg/kg) the combination of treatment produced a significant reversal of motor deficits as measured by peak scores (Fig. 5.7 D), but there was no effect on total scores (AUC_{0-5h}) and on time (Fig. 5.7 B & C).

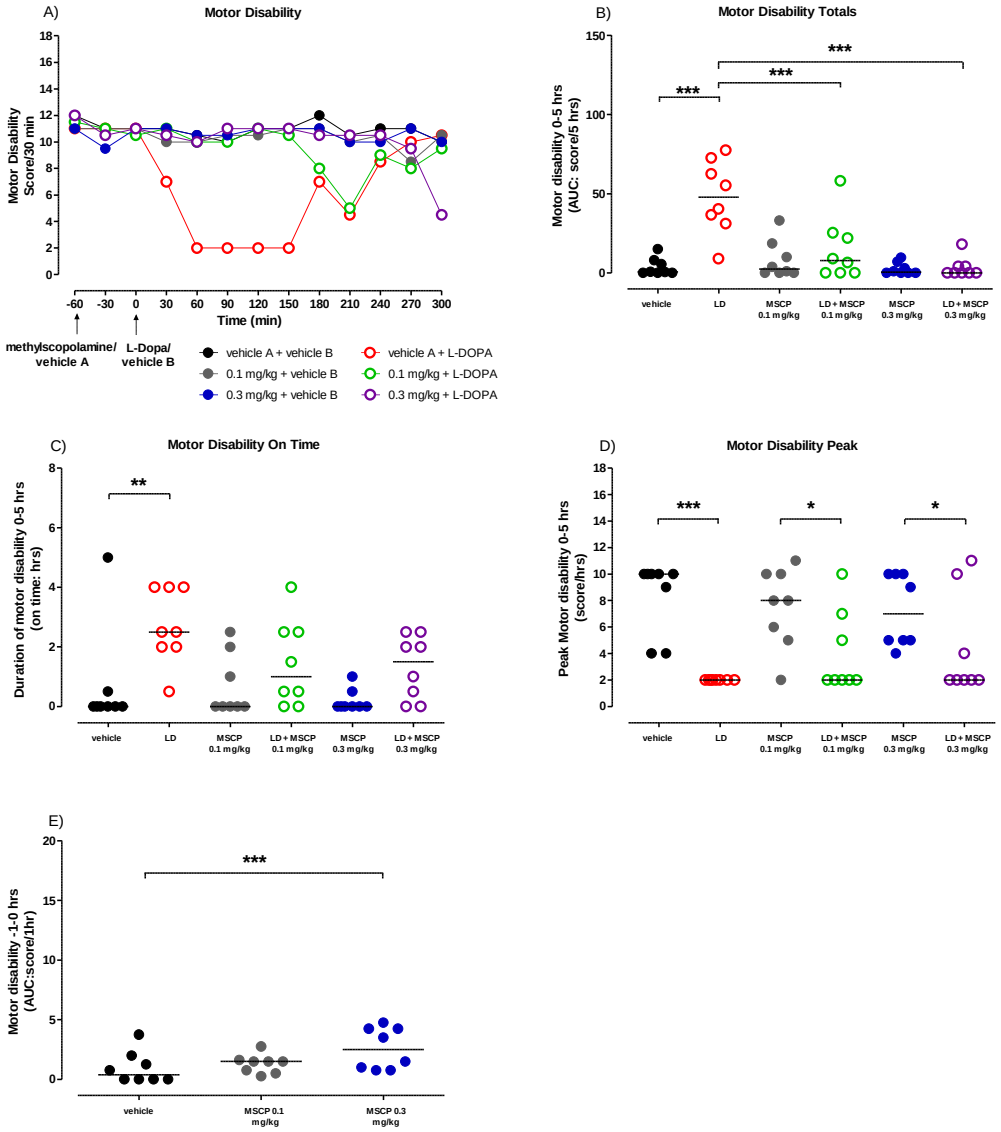


Figure 5.7 Effect of methylscopolamine on L-DOPA-induced motor disability in MPTP-treated common marmosets

Methylscopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Motor disability time course and B) Total motor disability reversal ($AUC_{0\text{-}5h}$) C) On-time and D) Peak motor disability after L-DOPA/vehicle-B administration; E) Total motor disability reversal in the first hour after methylscopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; For B, D & E data were transformed $y=\sqrt{y}$; (B) $F=16.81$; Df (5,47); $p<0.0001$; C) $F=5.616$; Df (5,47); $p=0.0007$; D) $F=8.896$; Df (5,47); $p<0.0001$; E) $F=4.483$; Df (2,23); $p=0.0313$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

5.3.2 Clinically used selective M1 anticholinergics

5.3.2.1 Trihexyphenidyl

5.3.2.1.1 Locomotor activity

Trihexyphenidyl alone (0.5 & 1 mg/kg) had no significant effect on locomotor activity as measured by total counts over the first hour (AUC_{-1-0h}) prior to L-DOPA administration compared to vehicle-A (Fig. 5.8 E). However, during the subsequent 5 hours trihexyphenidyl produced a significant increase in locomotor activity as measured by on time (1 mg/kg) when compared to vehicle-A/B-treated animals (Fig. 5.8 C).

Pre-treatment with trihexyphenidyl had no significant effect on L-DOPA-induced rise in locomotor activity as measured by total counts (AUC_{0-5h}) and peak activity (Fig. 5.8 B & D) but the highest dose of trihexyphenidyl (1 mg/kg) significantly increased duration of L-DOPA (Fig. 5.8 C) when compared to L-DOPA alone.

Combination treatment L-DOPA/trihexyphenidyl remained significantly improved as measured by total counts (AUC_{0-5h}), on-time and peak activity (Fig. 5.8 B – D) when compared to trihexyphenidyl alone.

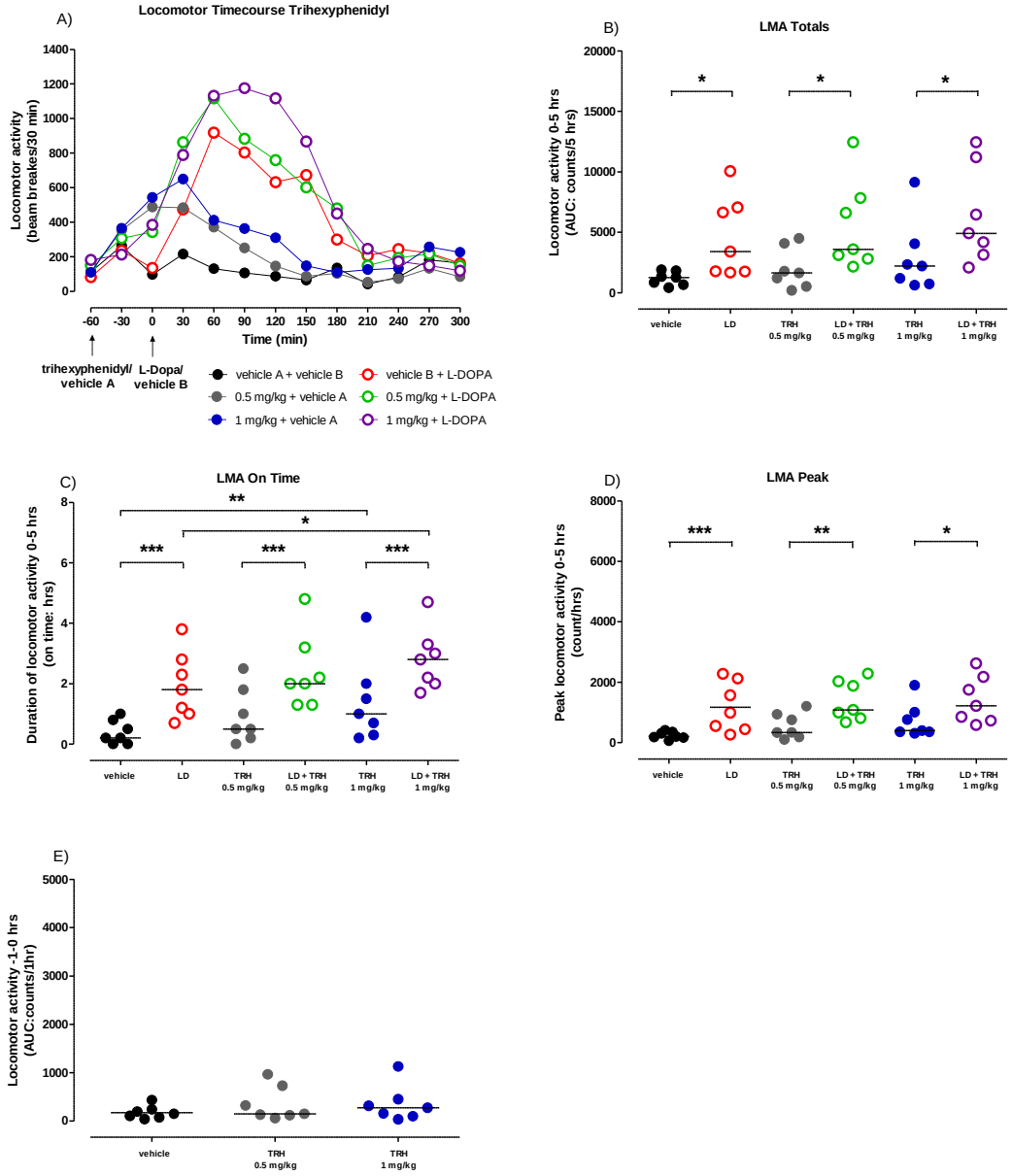


Figure 5.8 Effect of trihexyphenidyl on L-DOPA-induced locomotor activity in MPTP-treated common marmosets

Trihexyphenidyl (0.5 & 1 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Locomotor activity time course and B) Total locomotor activity ($AUC_{0\text{-}5h}$), C) On-time and D) Peak locomotor activity after L-DOPA/vehicle-B administration; E) Total activity in the first hour after methylscopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with mean values with error bars omitted for clarity (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; (B) F=7.578; Df (5,41); p=0.0001; C) F=15.72; (5,41); p<0.0001; D) F=11.00 (5,41); p<0.0001; E) F=1.788; (2,20); p=0.2091) followed by Newman-Keuls post hoc test *p<0.05; **p<0.01; ***p<0.001.

5.3.2.1.2 Motor disability

Trihexyphenidyl alone (0.5 & 1 mg/kg) had no effect on reversal of motor disability over the first hour as measured by totals (AUC_{-1-0h}) when compared to vehicle-A (Fig. 5.9 E), however, the highest dose of trihexyphenidyl (1 mg/kg) produced a significant improvement in motor disability over the 5 hours after the vehicle-B administration as measured by on-time and peak activity when compared to vehicle-A/B-treated animals (Fig. 5.9 C & D).

Pre-treatment with trihexyphenidyl (0.5 & 1 mg/kg) had no significant effect of on L-DOPA-induced reversal of motor disability as measured by totals scores (AUC_{0-5h}), however, the highest dose (1 mg/kg) produced a significant effect on duration and peak scores (Fig. 5.9 C & D) when compared to L-DOPA alone.

Reversal of motor deficits by L-DOPA/trihexyphenidyl combination remained significantly different to trihexyphenidyl alone as measured by on-time (Fig. 5.9 C) and at lower dose (0.5 mg/kg) as measured by peak (Fig. 5.9 D).

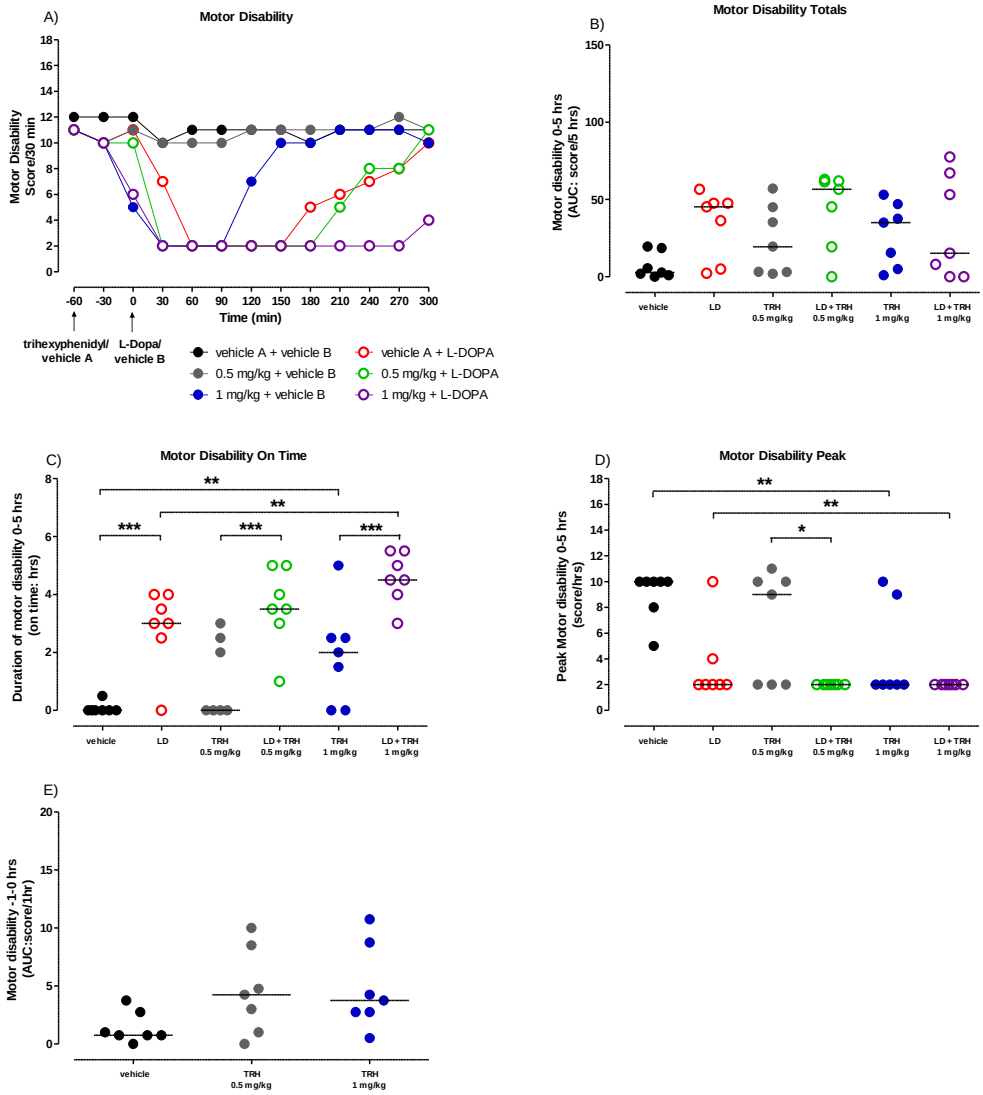


Figure 5.9 Effect of trihexyphenidyl on L-DOPA-motor disability in MPTP-treated common marmosets

Trihexyphenidyl (0.5 & 1 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Motor disability time course and B) Total motor disability ($AUC_{0\text{-}5h}$), C) On-time and D) Peak motor disability after L-DOPA/vehicle-B administration; E) Total motor disability in the first hour after trihexyphenidyl/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; For B, D & E data were transformed $y=\sqrt{y}$; (B) F=1.702; Df (5,41); p=0.1646; C) F=21.31; Df (5,41); p<0.0001; D) F=8.424; Df (5,41); p<0.0001; E) F=2.988; Df (5,41); p=0.0885) followed by Newman-Keuls post hoc test *p<0.05; **p<0.01; ***p<0.001.

5.3.2.2 Benztropine

5.3.2.2.1 Locomotor activity

Benztropine alone (0.25 & 0.5 mg/kg) produced an immediate increase in locomotor activity, which lasted for about 2.5 hrs, resulting in a significant increase in locomotor activity counts over the first hour (AUC_{-1-0h}) prior to L-DOPA/vehicle-B administration compared to vehicle-A (Fig. 5.10 E).

Pre-treatment with benztropine (0.25 & 0.5 mg/kg) had no effect on improvement in locomotor activity as measured by total counts (AUC_{0-5h}), on-time or peak activity when compared to L-DOPA alone (Fig. 5.10 B & D).

When compared to benztropine alone, the combination treatment L-DOPA/benztropine remained significant increased as measured total counts (AUC_{0-5h}) at the higher dose (0.5 mg/kg) and on-time (Fig. 5.10 B & C).

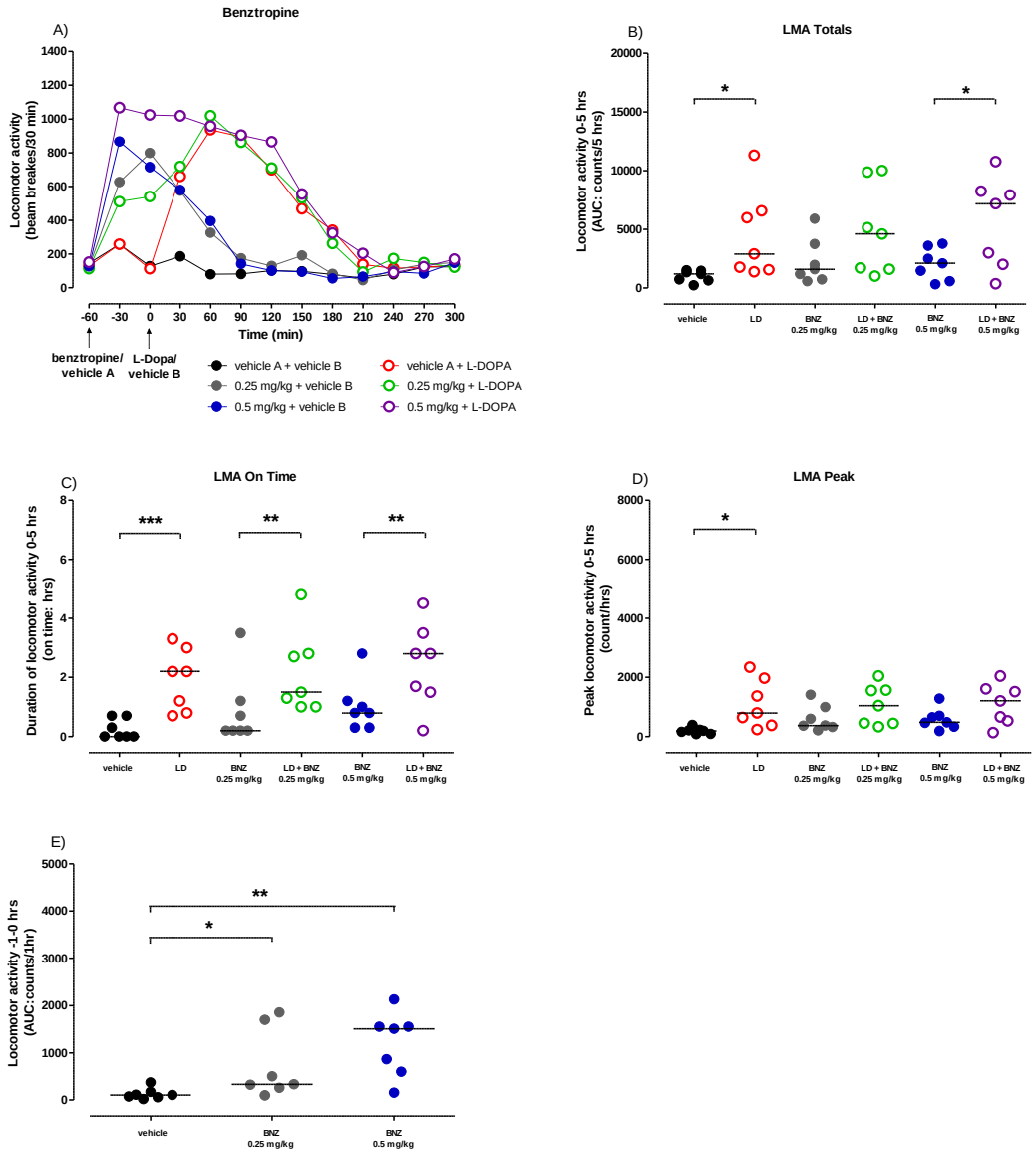


Figure 5.10 Effect of benztropine on L-DOPA-induced locomotor activity in MPTP-treated common marmosets

Benztropine (0.25 & 0.5 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Locomotor activity time course and B) Total locomotor activity ($AUC_{0\text{-}5h}$), C) On-time and D) Peak locomotor activity after L-DOPA/vehicle-B administration; E) Total locomotor activity in the first hour after benztropine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with mean values with error bars omitted for clarity (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; (B) F=6.180; (5,41); p=0.0005; C) F=10.68; (5,41); p<0.0001; D) F=3.082; (5,41); p=0.0204; E) F=10.08; (2,20); p=0.0027) followed by Newman-Keuls post hoc test *p<0.05; **p<0.01; ***p<0.001.

5.3.2.2.2 Motor disability

Benztropine alone (0.25 & 0.5 mg/kg) immediately and significantly reversed motor disability as measured by total scores over the first hour (AUC_{-1-0h}) prior to L-DOPA administration when compared to vehicle-A treatment (Fig. 5.11 E). This resulted in a significant improvement in motor disability over the 5 hrs after vehicle-B administration as measured by total (AUC_{0-5h}) scores, on-time and peak scores compared to vehicle-A/B treatment (Fig. 5.11 B – D).

There was no significant effect of benztropine (0.25 & 0.5 mg/kg) on L-DOPA-induced reversal of motor disability as measured by total (AUC_{0-5h}) and peak scores and on-time when compared to L-DOPA alone

Reversal of the motor disability by the L-DOPA/benztropine combination had no effect on total score (AUC_{0-5h}), on-time and peak score when compared to benztropine alone (0.25 & 0.5 mg/kg) (Fig. 5.11 B – D).

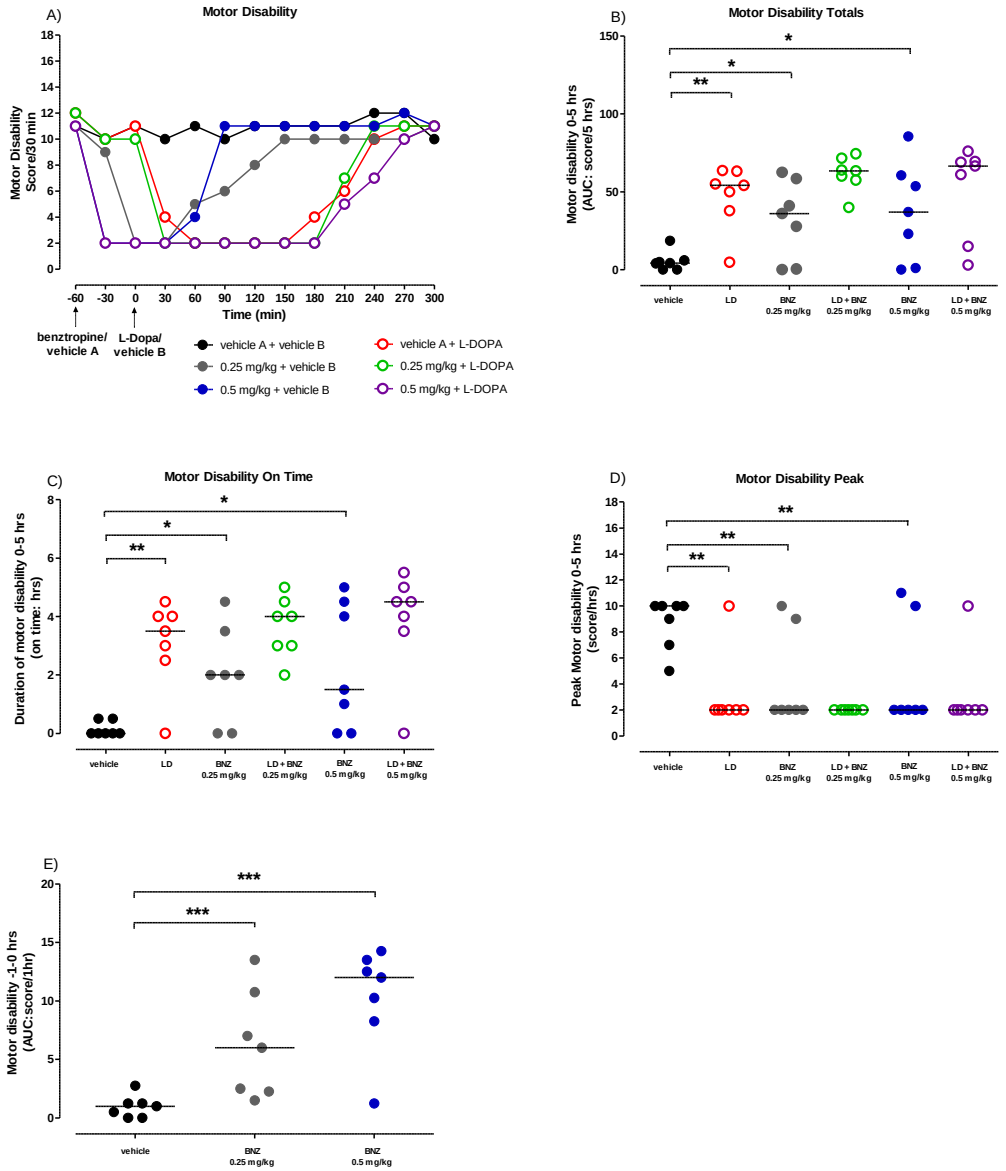


Figure 5.11 Effect of benztropine on L-DOPA-induced motor disability in MPTP-treated common marmosets

Benztropine (0.25 & 0.5 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Motor disability time course and B) Total motor disability ($AUC_{0\text{-}5h}$), C) On-time and D) Peak motor disability after L-DOPA/vehicle-B administration; E) Total motor disability in the first hour after benztropine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (n=7) (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; For B, D & E data were transformed $y=\sqrt{y}$; (B) $F=15.523$; Df (5,41); $p=0.0010$; C) $F=6.339$; Df (5,41); $p=0.0004$; D) $F=5.411$; Df (5,41); $p=0.0012$; E) $F=24.44$; Df (5,41); $p<0.0001$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

5.3.3 Selective M4 antagonist - NBI-675

5.3.3.1 Locomotor activity

NBI-675 (1 – 7.5 mg/kg) alone has not significantly improved locomotor activity as measured by totals ($AUC_{-1\text{-}0h}$) and ($AUC_{0\text{-}5.5h}$), on-time and peak activity when compared to vehicle-A/B (Fig. 5.12 B – E).

Pre-treatment with NBI-675 (1 – 7.5 mg/kg) had no effect on increase of locomotor activity when compared to L-DOPA-alone-treated animals (Fig. 5.12 B – E), although, the highest dose of NBI-675 (7.5 mg/kg) in combination with L-DOPA tended to produce a decrease in locomotor activity as measured by totals ($AUC_{0\text{-}5.5h}$) when compared to L-DOPA alone (Fig. 5.12 B).

When compared to NBI-675 alone, the combination treatment remained significantly increased as measured by total counts ($AUC_{0\text{-}5h}$) and peak activity at the lower doses (1 & 5 mg/kg), whereas on time tended to be increased (Fig. 5.12 B – D).

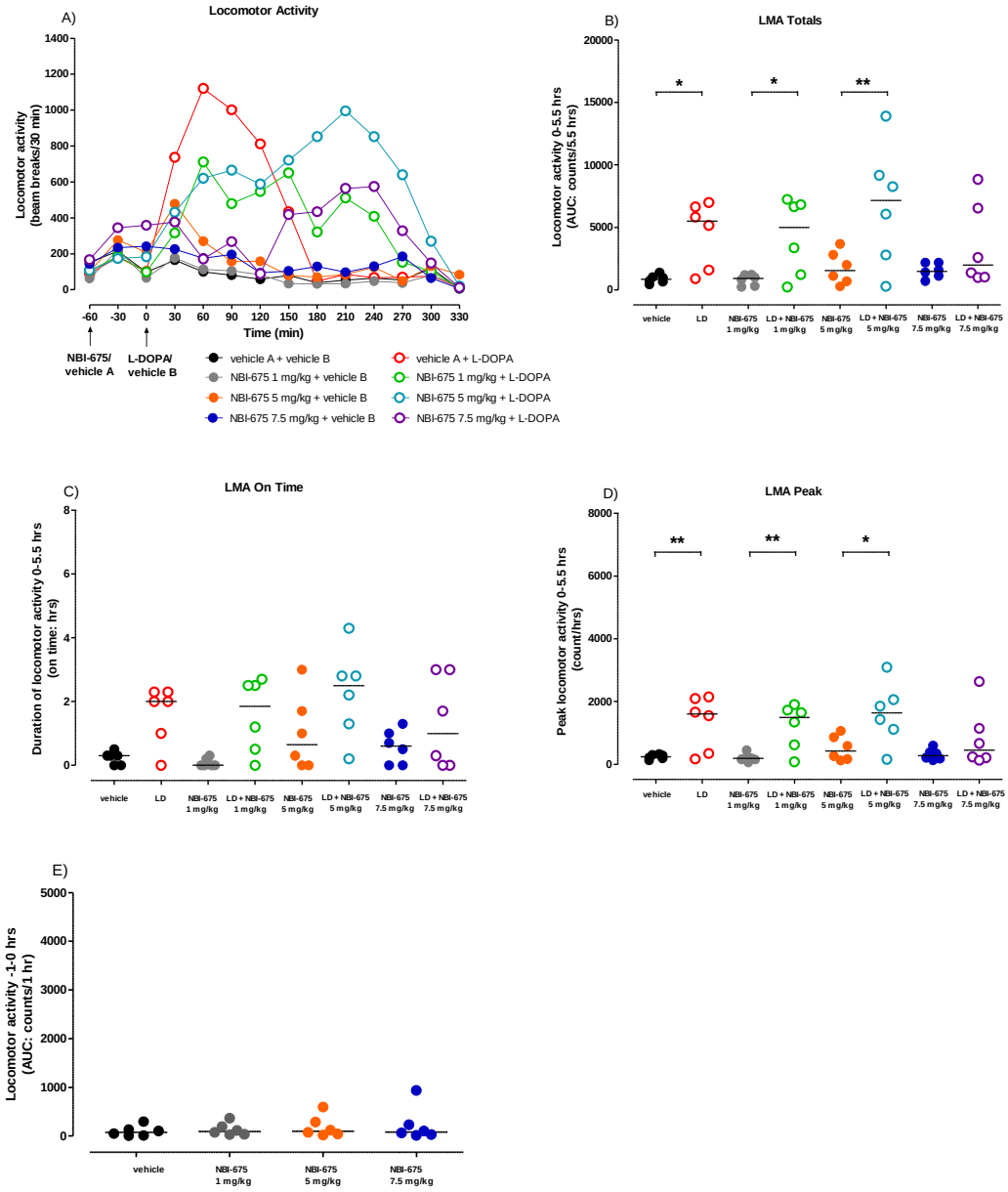


Figure 5.12 Effect of NBI-675 on L-DOPA-induced locomotor activity in MPTP-treated common marmosets

NBI-675 (1 – 7.5 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=6) A) Locomotor activity time course and B) Total locomotor activity ($AUC_{0\text{-}5h}$), C) On-time and D) Peak locomotor activity after L-DOPA/vehicle-B administration; E) Total locomotor activity in the first hour after NBI-675/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with mean values with error bars omitted for clarity (n=6) (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; (B) F=6.309; Df (7,47); p<0.0001; C) F=4.029; Df (7,47); p=0.0025; D) F=7.316; Df (7,47); p<0.0001; E) F=0.9252; Df (3,23) p=0.4526) followed by Newman-Keuls post hoc test *p<0.05; **p<0.01.

5.3.3.2 Motor disability

NBI-675 alone (1 – 7.5 mg/kg) had no significant effect on reversal of motor disability totals (AUC_{-1-0h}) and ($AUC_{0-5.5h}$), on-time and peak activity when compared to vehicle-A treatment (Fig. 5.13 B – E).

Similarly, NBI-675 had no significant effect on L-DOPA-induced reversal of motor disability when compared to L-DOPA alone (Fig. 5.13 B – D). The combination treatment NBI-675/L-DOPA showed significant reduction on reversal of motor deficits when measured by peak at the highest dose (7.5 mg/kg), while on time tended to be increased when compared to NBI-675 alone treatment (Fig. 5.13 C).

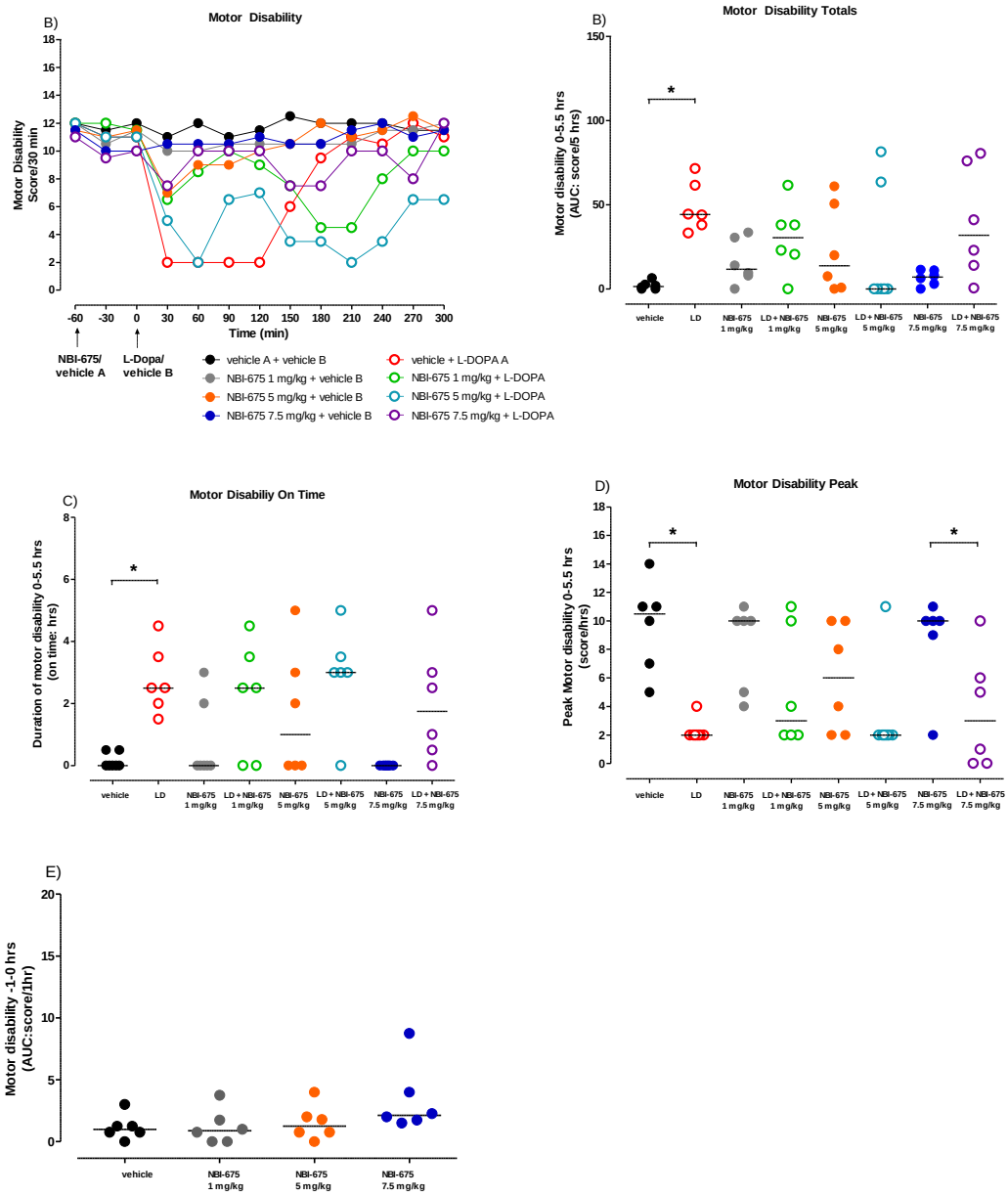


Figure 5.13 Effect of NBI-675 on L-DOPA-induced motor disability in MPTP-treated common marmosets

NBI-675 (1 – 7.5 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=6) A) Motor disability time course and B) Total motor disability ($AUC_{0\text{-}5h}$), C) On-time and D) Peak motor disability after L-DOPA/vehicle-B administration; E) Total motor disability in the first hour after NBI-675/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (n=6) (A) and median with individual counts (B – E). A) No statistical analysis performed; B – D) Data analysed by Two way ANOVA and B – E) repeated measures ANOVA; For B, D & E data were transformed $y=\sqrt{y}$; (B) F=2.773; Df (7,47); p=0.0211; C) F=4.055; Df (7,47); p=0.0024; D) F=4.132; Df (7,47); p=0.0021; E) F=3.431; Df (3,23); p=0.0444) followed by Newman-Keuls post hoc test *p<0.05.

5.3.4 The effect of NBI-675 on L-DOPA and its metabolite

Analysis of blood plasma showed no effect of NBI-675 (5 mg/kg p.o.) on concentration of L-DOPA (Fig. 5.14 A) and its metabolite 3-OMD (Fig. 5.14 B) at 1 hr and 3 hr after L-DOPA administration, however, the concentration of L-DOPA was significantly lower at 3 hr time point when compared to 1 hr, whereas concentration of 3-OMD was significantly higher at 3 hr when compared to 1 hr.

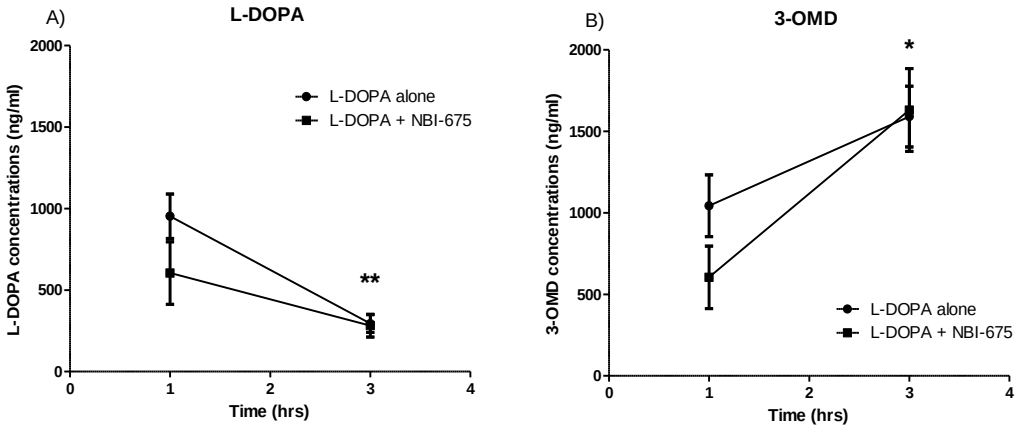


Figure 5.14 Effect of NBI-675 administration on A) L-DOPA and B) 3-OMD levels in common marmoset blood plasma following 1 and 3 hours after L-DOPA administration
Concentrations of L-DOPA and 3-OMD in blood plasma were determined using HPLC-ECD. Data are presented as mean ± SEM (n=6); A) **p<0.01 L-DOPA alone (4 mg/kg + benserazide 10 mg/kg p.o.) B) *p<0.05 L-DOPA (4 mg/kg + benserazide 10 mg/kg p.o.) + NBI-675 (5 mg/kg p.o.) (Two-way ANOVA and Friedman's test followed by post hoc Dunn's test (A) FS = 12.60; p=0.0015; B) FS = 10.80; p=0.0061).

5.4.4 Other effects of anticholinergics

The primary aim of the study was to investigate the effects of anticholinergics on motor function in MPTP-treated common marmosets, therefore other central and peripheral effects of anticholinergics were not assessed quantitatively. However, based on the observation of animals through a one-way mirror during a scoring period, animals administered with high doses of anticholinergics appeared to be disorientated at times with reduced activity and movement, recumbent and somnolent, with occasional tracking particularly within the first hour of drugs administration. These effects are similar to those reported in the rodent (Chapter 3 & 4) and are a typical CNS effects

of anticholinergic treatment, which are important dose limiting considerations in clinical use. Additionally, high doses of NBI-675 produced nausea and vomiting in some animals within the first hour of administration. In addition, one animal had seizure on the highest dose of the compound, thus had to be taken out of the study.

5.4 Discussion

The studies described in this chapter explored the effect of centrally acting anticholinergics in PD with and without L-DOPA in the MPTP-treated marmosets, with the particular emphasis on selective inhibition of M4 muscarinic receptors. It was hypothesised that selective antagonism of muscarinic M4 receptors with NBI-675 will relieve motor symptoms of Parkinson's disease when given alone and in combination with L-DOPA.

L-DOPA has remained the gold standard treatment for PD (Rascol *et al.*, 2000) and so as expected these studies confirmed that L-DOPA alone almost immediately attenuated bradykinesia produced by MPTP-treatment increasing locomotor activity and reversing motor symptoms observed in MPTP-treated marmosets.

Centrally acting non-selective scopolamine and M1 selective trihexyphenidyl and benztropine tended to improve locomotion and reverse motor deficits in MPTP-treated marmosets when administrated alone and in combination with L-DOPA. Interestingly, peripherally acting methylscopolamine produced immediate and very short-lasting reversal of motor deficits, but overall unexpectedly it showed a tendency to antagonize L-DOPA reversal of motor disability. Selective M4 antagonist, NBI-675, had no beneficial effect on improvement of locomotion or motor disability either alone or when combined with L-DOPA. All anticholinergics appeared to reduce maximal L-DOPA activity while extending its duration, which might be clinically useful. This could also indicate, that they may delay absorption of L-DOPA, nevertheless, undertaken pharmacokinetic analysis did not confirm this postulation.

5.4.1 The effect of anticholinergics on motor symptoms in MPTP-treated marmosets

Although the use of anticholinergics in the symptomatic treatment of PD have been supplanted by more efficacious dopamine agonists and L-DOPA, anticholinergics are still frequently prescribed, mainly in the early stages of the disease, where tremor is predominant and in PD-related dystonia (Brocks, 1999; Fox *et al.*, 2011). Despite this, the occurrence of often unpleasant and severe side effects limits their use, as the currently used compounds are non-selective for the specific subtype of muscarinic receptor. The imbalance between the striatal DA and ACh systems has long been

implicated in the regulation of movement disorders (Pisani *et al.*, 2007; Aosaki *et al.*, 2010; Lester *et al.*, 2010) and preclinical animal studies, including those described in this thesis and in previous publications, have indicated involvement of striatal muscarinic M4 receptors in the regulation of motor control (Mayorga *et al.*, 1999; Karasawa *et al.*, 2003; Betz *et al.*, 2007). For this reason initially the effects of centrally acting anticholinergics were investigated on the improvement of motor symptoms when administered alone, without L-DOPA, in MPTP-treated marmosets with a view to establishing if indeed the M4 receptor is a viable target. The antimuscarinics used in the current study are used clinically and included the non-selective antagonist (scopolamine), and the M1 selective (trihexyphenidyl and benztropine). Generally, the results demonstrate that these antimuscarinics scopolamine, trihexyphenidyl and benztropine improved locomotion and reduced motor disability seen in animals treated with the MPTP, which is in agreement with previously published studies (Close *et al.*, 1990; Jackson *et al.*, 2014), and with respect to the latter, reflect their clinical use. The findings from previous rodent studies indicate that in general anticholinergic drugs increase locomotor activity (Crofton *et al.*, 1991; Sipos *et al.*, 1999). Scopolamine reversed motor disability, whereas methylscopolamine failed to show any effect on parkinsonian symptoms, indicating the central involvement in this process as shown previously in the rodent studies (Chapter 3). This is also in agreement with previously conducted studies in our lab (Jackson *et al.*, 2014). Despite all the positive effect of centrally acting drugs described above, the M4 selective NBI-675 antimuscarinic failed to ameliorate parkinsonian symptoms. The reasons for the lack of effect of selective muscarinic M4 antagonist in modulation of motor dysfunction in PD is not clear. Inhibitory M4 receptors are predominantly expressed postsynaptically on D1-MSN where they show inhibitory control on D1-mediated locomotor stimulation (Gomeza *et al.*, 1999a; Santiago & Potter, 2001; Pisani *et al.*, 2007). However, M4 are also located presynaptically, together with M2 receptors on ChI terminals, and they function as autoreceptors inhibiting ACh release (Kreitzer, 2009). It has been reported that rather than causing an increase of ACh release by loss of D2 inhibition, the loss in striatal dopamine reduces M4 autoreceptor signalling in the ChI following attenuation of M4 muscarinic autoreceptor coupling to Cav2 Ca2+ channels (and K+ channels) regulating acetylcholine release and spiking (Ding *et al.*, 2006). Therefore, the antagonism of M4 autoreceptors could result in proparkinsonian rather than

antiparkinsonian effect in the case of striatal DA denervation. However, in the present study this pro-parkinsonian effect was not observed suggesting that other M4 mediated pathways are affected. In the parkinsonian state, the direct pathway is hypoactive due to loss of D1 activation, whereas the indirect pathway is hyperactive due to the loss of D2 inhibition. It has been previously shown by using selective M1 antagonists, that inhibition of excitatory M1 receptors located on both the direct and indirect MSN, can inhibit activity of both output pathways (Ding *et al.*, 2011; Erosa-Rivero *et al.*, 2014) thus opposing the loss of DA on the direct pathway, but enhancing its effect on the indirect pathway. While non-M4 selective compound improved locomotion and parkinsonian symptoms in this study using MPTP-treated marmoset model, therefore perhaps M1 receptors are the subtypes of receptors as antiparkinsonian drug targets. However, targeting the M1 receptors, may result in severe side effect, due to their location in the CNS and periphery, as described in Chapter 1.

5.4.2 The effect of anticholinergics on L-DOPA-induced motor symptoms in MPTP-treated marmosets

The effect of pre-treatment with anticholinergics on L-DOPA-induced improvement of parkinsonian symptoms was also investigated to assess whether they would alter the L-DOPA response. All clinically used centrally and peripherally acting anticholinergics reduced the peak effect of L-DOPA resulting in a decrease of maximal activity while increase in locomotor activity was extended, which agrees with previous observations (Jackson *et al.*, 2014). These results are in agreement with the limited number of clinical studies where the overall improvement in tremor, bradykinesia and rigidity on the comparison of anticholinergic and dopaminergic therapy was reported (Parkes *et al.*, 1974; Koller, 1986). In addition, centrally acting anticholinergics in combination with L-DOPA improved parkinsonian disability, whereas peripherally acting methylscopolamine appeared to block the L-DOPA improvement of motor deficits. This effect is unexpected to be seen since the brain penetration of methylscopolamine is rather poor (Crofton *et al.*, 1991), although studies on squirrel monkey have shown that methylscopolamine can cross the BBB, but at the dose about 10 times higher to that of scopolamine (Pakarinen & Moerschbaecher, 1993), so unlikely to be of importance in this study. Moreover, Crofton and colleagues (1991) compared studies from various laboratories utilizing different devices to monitor

motor activity and the effect of numerous drugs on motor functions in rats. Among many, they found that in majority of the tests, methylscopolamine showed either no effect on motor activity or small non-dose dependent decrease (Crofton *et al.*, 1991). The possible explanation to the effect seen in these studies could involve the strong inhibitory effect of methylscopolamine on peripherally located muscarinic receptors.

The selective M4 antagonist NBI-675 did not improve the L-DOPA-induced antiparkinsonian effect nor reversal of motor deficits in MPTP-treated marmosets, indeed, the highest dose of the NBI-675 tended to suppress the L-DOPA activity. The reason for this is unclear, but could be a consequence of its effect on the CNS. It has been reported and it is well known that anticholinergics cause many central side effects, including drowsiness or confusion (Wawruch *et al.*, 2012), making animals less able to conduct motor movement. Additionally, higher doses of the compound might have led to loss of receptor selectivity. As it was observed in rat studies in this thesis (Chapter 3 and 4) higher doses of anticholinergics, including NBI-675, produced somnolence, recumbency and overall reduced motion. Cognitive side effects, including sedation and memory impairments, are important dose limiting issues in human use. There is a number of assessments which could be used to test learning and memory in animals, and these include Morris water maze or Y-maze (T-maze), used mainly in rodents, while Wisconsin General Test Apparatus (WGTA) was mainly designed to be used in non-human primates.

The failure of the improvement of parkinsonian disability in the current study could perhaps be related to the *in vitro* affinity constant of the compound as the selectivity *in vivo* could have changed and could be different in living cells and isolated membranes (Saunders *et al.*, 1996). However, if this would be the case, the compound would have not been effective in blocking the pilocarpine-induced chewing movements in Chapter 3.

With the overall improvement of motor function evoked by anticholinergic drugs, the duration of the L-DOPA effect was prolonged, and we proposed that was caused by decrease in L-DOPA absorption due to a delay in gastric emptying of L-DOPA in plasma. The results correlate closely to that seen in man and reported previously in both preclinical and clinical studies which have indicated that combination of anticholinergics with L-DOPA could alter the plasma concentration of L-DOPA

(Algeri *et al.*, 1976; Contin *et al.*, 1991; Djaldetti *et al.*, 1996; Roberts *et al.*, 1996). As seen with the clinically used anticholinergics, NBI-675 also delayed the decrease in peak effect of L-DOPA and prolonged duration of activity, the PK study of blood plasma resulted in no significant effect of the NBI-675 on L-DOPA concentration, indicating that the antimuscarinic has no effect on L-DOPA concentration in the blood plasma. Perhaps this could be caused by small sample size, or not sufficient time points of blood samples.

5.5 Conclusion

In conclusion, this study showed that cholinergic system plays a role in mediation of motor control, however, the mechanism is not entirely clear. Contrary to the results obtained in the Chapter 3 on the effect of the selective M4 antagonist in reduction of involuntary movements, the hypothesis of this chapter is rejected, since the selective muscarinic M4 antagonist NBI-675 has shown no effect in the improvement of parkinsonian disability produced by MPTP treatment in the common marmoset. However, since the NBI-675 suppressed involuntary movements in rats, as shown in Chapter 3, therefore it is important to test whether this inhibitory effect would be replicated in higher species, where dyskinesia, and particularly dystonia, is a debilitating symptom of L-DOPA therapy. Therefore, the subsequent study will explore, whether administration of NBI-675 to MPTP-treated common marmoset will alter L-DOPA-induced dyskinesia.

Chapter 6 The effect of anticholinergic therapy on L-DOPA-induced dyskinesia in MPTP-treated common marmosets

6.1. Introduction

In the preceding chapter it was reported that selective inhibition of muscarinic M4 receptors was ineffective in improvement of motor symptoms in MPTP-treated marmoset when administered alone or in combination with L-DOPA. However, since the results from Chapter 3 indicate that muscarinic M4 receptors are involved in the mediation of involuntary movements in rats, and substantial evidence from the literature that cholinergic system plays a role in dyskinesia (Bezard *et al.*, 2001), it is important to test whether selective muscarinic M4 inhibition would alter L-DOPA-induced dyskinesia in the MPTP-treated marmoset.

As previously described (Chapter 2) MPTP-treated primate model is highly reproducible and reflects symptoms and neurophysiological changes observed in man (Fox *et al.*, 2011). Chronic L-DOPA treatment results in the onset of L-DOPA-induced dyskinesia (LID), including chorea, dystonia and athetosis, closely resembling dyskinesia seen in idiopathic PD (Burns *et al.*, 1983; Jenner, 2003a), thus this model closely mimics these characteristics of drug treatment that appears in man. LID have high prevalence, being observed in approximately 40% of patients within 5 years of initiation of L-DOPA, rising to approximately 90% after 10 years (Ahlskog & Muenter, 2001; Fabbrini *et al.*, 2007). In some cases, LID can be more debilitating than the disease itself and in many patients significantly may affect quality of life. For this reason, it is essential to find alternative treatment strategies that could reduce the expression of dyskinesia.

Pathological alterations in the striatal ACh signalling are associated with the expression of dyskinesia (Ding *et al.*, 2006; Ding *et al.*, 2011). Currently, anticholinergics are used in PD primarily for the treatment of tremor and dystonia in PD (Fox *et al.*, 2011), although the dystonia differs to the idiopathic type previously discussed (Chapter 3). It can be divided into "off" and "on" dystonia. "Off" dystonia usually manifests either in the early morning or during the "wearing off" of L-DOPA (Poewe *et al.*, 1988). "On" dystonia is seen as a part of L-DOPA-induced dyskinesia and correlates with antiparkinsonian effect once the patient is primed.

Previous studies have demonstrated beneficial effect of anticholinergics in the treatment of dystonia in PD (Pourcher *et al.*, 1989), however, there has been a number of reports in PD patients showing that anticholinergic treatment can trigger dyskinesia,

mainly oro-buccal dystonia (Hauser & Olanow, 1993) as well as choreic movements (Linazasoro, 1994) and abnormal movements of limbs (Birket‐Smith, 1974).

The role of anticholinergics in LID in animal models of PD is equivocal as there is limited number of studies looking at the effects of anticholinergics and LID. It has been reported that muscarinic antagonist dicyclomine reduced expression of dyskinesia in a mouse model of LID (Ding *et al.*, 2011). By contrast, studies in non-human primates show that non-selective antimuscarinics had no effect on overall expression of LID in MPTP-treated primates, although the drugs altered the nature of dyskinesia from dystonia to chorea (Gomez-Mancilla & Bedard, 1993; Jackson *et al.*, 2014). Despite the mixed reports, these findings support the idea of an involvement of cholinergic transmission in mediation of expression of LID. However, there was no attempt to interrogate the subclass of muscarinic receptor involved, as the antimuscarinics used in these studies were non-selective. The finding that the M4 antagonist NBI-675 reduced the expression of pilocarpine-induced perioral movements suggests a role in reducing dystonia expression in PD. For this reason, for the first time, the effect of selective antagonism of the M4 muscarinic receptor on the expression of L-DOPA-induced dyskinesia (dystonia/chorea) has been investigated in MPTP-treated marmoset previously primed to L-DOPA.

6.1.1. Hypothesis

It is hypothesised that selective antagonism of muscarinic M4 receptors will reduce the expression of L-DOPA-induced dyskinesia and dystonia.

6.1.2. Aims

Specific aims of this study were to:

1) Confirm that L-DOPA induces dyskinesia, including dystonia and chorea, in MPTP-treated common marmosets.
2) Determine the role of anticholinergics of different selectivity to muscarinic receptor on expression of L-DOPA-induced dyskinesia, including dystonia and chorea, and specifically the role of selective M4 muscarinic receptors antagonist, NBI-675, in MPTP-treated primates.

6.2 Materials and Methods

In order to address these aims the following studies were performed:

1) The effect of L-DOPA on the expression of dyskinesia was determined in MPTP-treated common marmosets previously primed to exhibit dyskinesia by chronic L-DOPA therapy.
2) The role of clinically used anticholinergics alone and their effect on L-DOPA-induced expression of dyskinesia (dystonia and chorea) was investigated by treating MPTP-treated common marmosets with peripheral and central acting anticholinergics 1 hr prior to L-DOPA or vehicle. Changes in expression of dyskinesia (dystonia and chorea) were assessed to determine drug effect.
3) The role of the M4 muscarinic receptor in L-DOPA-induced expression of dyskinesia (dystonia and chorea) was investigated using the novel selective M4 antagonist NBI-675 administered to MPTP-treated common marmosets 1 hr prior to L-DOPA or vehicle. Changes in expression of dyskinesia (dystonia and chorea) were assessed to determine drug effect.

An overview is described below, but detailed methodology are to be found in Chapter 2.

6.2.1 Animals

Adult common marmosets (*Callithrix Jacchus*) (Harlan, UK, 350 – 500 g, n = 6-8 per group) of either sex, previously treated with MPTP as described in Chapter 2 section 2.2.3.1 were used in this study. Animals were housed singly or in pairs (mixed ♂♀or single sex ♀♀) in controlled environment suitable for the species (temperature 25 ± 1°C with 50% relative humidity on a 12 hour light/dark cycle). All animals had *ad libitum* access to water and Mazuri food pellets and received two meals each day. Animals were previously primed with L-DOPA and were not drug naïve prior to the study. Full details are described in section 2.2.3.1 Chapter 2.

All experiments were carried out in accordance with Home Office regulations under the Animas (Scientific Procedures) Act 1986 and project licence number 70/7146 and 70/8541.

6.2.2 Behavioural assessment

As described in Chapter 2, all behavioural assessments were carried out between the hours 7.00 am and 3.00 pm. Animals were given 60 min acclimatization in the test cages prior to drug treatment. Dyskinesia with dystonia and chorea assessment was assessed and scored at the same time as motor disability, and performed for 1 hr before (baseline score) and 6 hr after anticholinergics/vehicle administration, by experienced observers blinded to the drug treatment through one-way mirror during the last 10 min consecutive 30 min intervals. Full description of assessment criteria are provided in Chapter 2 section 2.2.3.3.

Locomotor activity, motor disability and dyskinesia with dystonia and chorea, were assessed during one experiment and on the same animals, however, for the clarity, behavioural outcomes were split into two chapters to assess the effects of anticholinergics on motor function (Chapter 5) and on L-DOPA-induced dyskinesia (Chapter 6).

6.2.3 Drug treatment

Full details of drug preparation and treatment are described in Chapter 2 section 2.2.3.4. Briefly, animals were placed into the testing units. Following the 60 min acclimatisation period which provided baseline activity data, animals were dosed with appropriate anticholinergic or vehicle (s.c. or p.o.) followed by L-DOPA + benserazide or vehicle 60 min later. Doses of anticholinergics are presented in Table 6.1. Behavioural assessment was then carried out as described in Chapter 2 section 2.2.3.3. A repeated crossover design was used, so each marmoset was treated with all doses of a single drug or vehicle with at least 72 hr wash-out between the tests. A typical modified Latin square was used to randomise drug treatments and is shown in Chapter 2 Table 2.2.

Dyskinesia with dystonia and chorea was scored immediately before each drug treatment and then every 30 minutes for 10 minute periods throughout the study for up to 5 hours, as shown in Chapter 2 Figure 2.7.
One animal had to be taken out of the study due to seizures caused by the highest dose of NBI-675.

Table 6.1 Doses and route of administration of drugs used in the study.

Drug	Dose and route
Benztropine	0.25 & 0.5 mg/kg s.c.
Scopolamine	0.1 & 0.3 mg/kg s.c.
Methylscopolamine	0.1 & 0.3 mg/kg s.c.
Trihexyphenidyl	0.5 & 1 mg/kg p.o.
NBI-675	1, 5 & 7.5 mg/kg p.o.
L-DOPA + benserazide	8 mg/kg p.o.+ 10 mg/kg p.o.

6.2.4 Statistical analysis

Data and statistical analysis were performed using GraphPad Prism 5.02 (San Diego, CA, USA) as described in Section 2.2.3.5, Chapter 2. The statistical analysis for dyskinesia, dystonia and chorea data was performed as follows:

1) No statistical analysis was performed on time course data;
2) Totals (AUC_{-1-0h}) and (AUC_{0-5h}) and Peak data were transformed by square root ($Y=\sqrt{Y}$).
3) Totals (AUC_{-1-0h}) and (AUC_{0-5h}), Peak, On-time, On-time > 2 were analysed by repeated measures ANOVA followed by *post hoc* Newman-Keuls multiple comparisons test.

6.3 Results

In all studies vehicle-A-vehicle-B administration had no effect on induction of dyskinesia, dystonia and chorea over time in MPTP-treated common marmosets (Fig. 6.1 – 6.15).

As described in section 5.3, following the acclimatisation time, and in the absence of L-DOPA, animals appeared akinetic and hunched, either sitting on the cage floor or on the perches. They were often staring in one direction, and at times looked sleepy, however, occasional alertness with head checking and overall slow movements were also present. Their locomotor activity scores were low with little variability (as described in Section 5.3) reflecting the akinesia, and their motor disability scores were high, as expected for a parkinsonian marmoset. Their dyskinesia (t=-1-0, median 0; range 0 – 4), dystonia (t=-1-0, median 0; range 0 – 4) and chorea (t=-1-0, median 0; range 0 – 3).

As expected, administration of L-DOPA alone induced moderate to severe dyskinesia, dystonia and chorea. The duration of L-DOPA-induced dyskinesia was between 3 and 3.5 hrs with a peak effect ranging between 30 min to 2 hrs after L-DOPA administration. This resulted in a significant induction of dyskinesia as measured by increased total scores ($AUC_{0\text{-}5h}$) (Fig. 6.1 B, 6.4 B, 6.7 B, 6.10 B & 6.13 B), on-time (Fig. 6.1 C, 6.4 C, 6.7 C & 6.10 C), and on-time > 2 (Fig. 6.1 D, 6.4 D & 6.7 D) and peak (Fig. 6. 1 E, 6.4 E, 6.7 E, 6.10 E & 6.13 E) when compared to vehicle-B-treated animals.

The duration of L-DOPA-induced dystonia was between 2.5 and 3.5 hrs with a peak at 1 to 2 hrs after L-DOPA administration. This produced a significant induction of dystonia as measured by total scores ($AUC_{0\text{-}5h}$) (Fig. 6.2 B, 6.5 B, 6.8 B & 6.11 B), on-time (Fig. 6.2 C, 6.5 C, 6.8 C & 6.11 C), on-time > 2 (Fig. 6.2 D & 6.5 D) and peak (Fig. 6.2 E, 6.5 E, 6.8 E & 6.11 E) when compared to vehicle-B treatment.

Similarly, the duration of L-DOPA-induced chorea was between 2.5 and 3 hrs with a peak at 30 min to 1.5 hrs after L-DOPA administration. This resulted in a significant induction of chorea as measured by total scores ($AUC_{0\text{-}5h}$) (Fig. 6.3 B, 6.6 B, 6.9 B, 6.12 B & 6.15 B), on-time (Fig. 6.3 C, 6.6 C, 6.9 C, 6.12 C & 6.15 C), on-time > 2

(Fig. 6.3 D & 6.6 D) and peak (Fig. 6.3 E, 6.6 E, 6.9 E, 6.12 E & 6.15 E) when compared to vehicle-B treatment.

6.3.1 The effect of non-selective anticholinergics

6.3.1.1 Centrally acting scopolamine

6.3.1.1.1 Dyskinesia

Scopolamine alone (0.1 & 0.3 mg/kg) produced an immediate and significant induction of dyskinesia over the first hour of treatment (AUC_{-1-0h}) (Fig. 6.1 F). This resulted overall in a significant increase in totals (AUC_{0-5h}), on-time and peak scores after the L-DOPA/vehicle administration when compared to vehicle-A treatment (Fig. 6.1 B, C & E).

Pre-treatment with scopolamine significantly increased the duration of L-DOPA-induced dyskinesia as measured by on-time and on-time > 2 when compared to L-DOPA alone (Fig. 6.1 C & D), although there was no significant effect of scopolamine on L-DOPA-induced total (AUC_{0-5h}) scores and peak (Fig. 6.1 B & E).

When compared to scopolamine alone, the combination L-DOPA/scopolamine remained significantly greater as measured by totals (AUC_{0-5h}), on-time and on-time > 2 scores (Fig. 6.1 B, C & D).

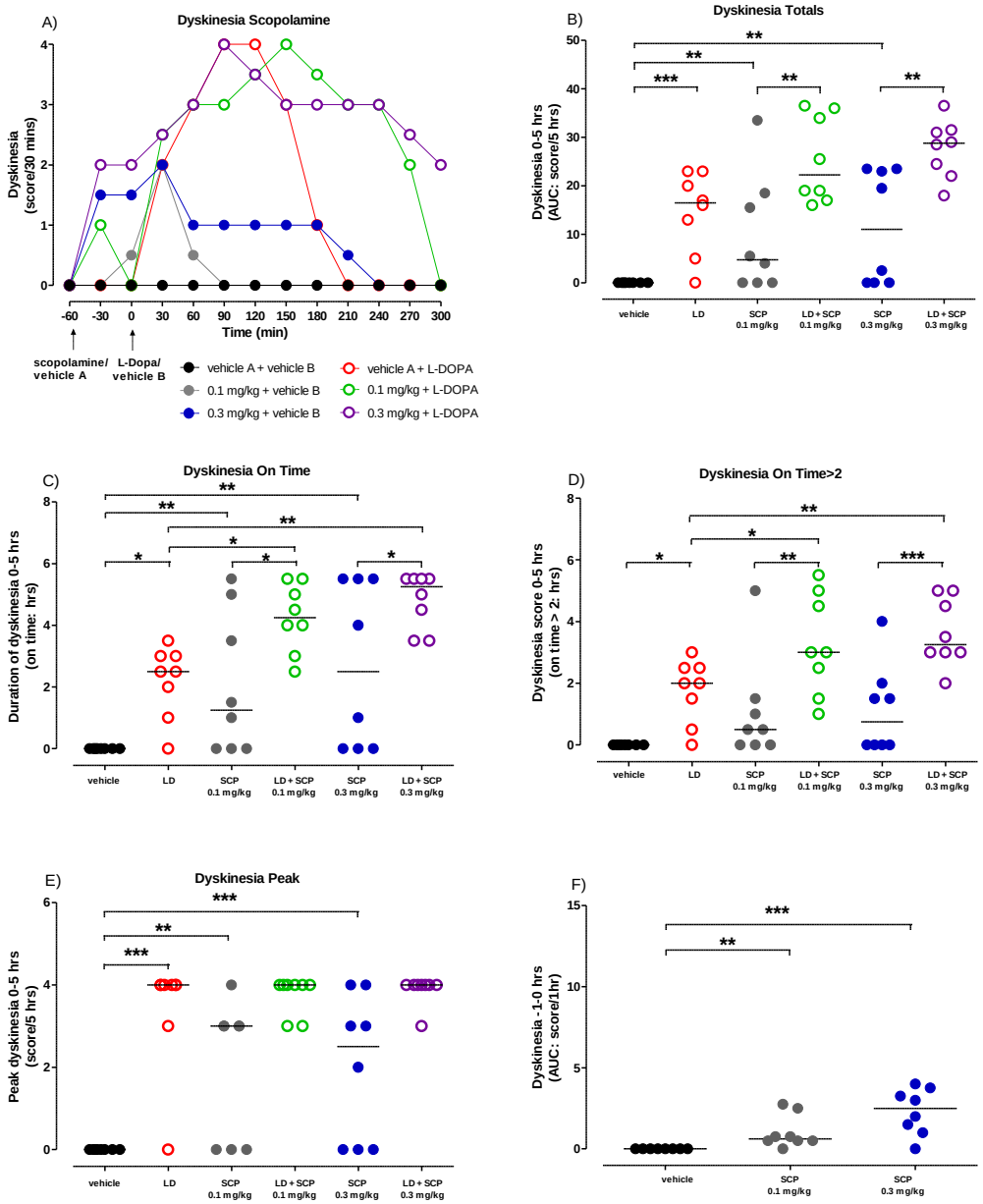


Figure 6.1 Effect of scopolamine on L-DOPA-induced dyskinesia in MPTP-treated common marmosets

Scopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Dyskinesia time course and B) Total dyskinesia ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak dyskinesia after L-DOPA/vehicle-B administration; F) Total dyskinesia in the first hour after scopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=14.89$; Df (5,47); $p<0.0001$; C) $F=11.12$; Df (5,47); $p<0.0001$; D) $F=12.66$; Df (5,47); $p<0.0001$; E) $F=12.41$; Df (5,47); $p<0.0001$; F) $F=21.05$; Df (2,23); $p<0.0001$) followed by Newman-Keuls post hoc test. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.1.1.2 Dystonia

As with dyskinesia, administration of scopolamine alone (0.1 & 0.3 mg/kg) produced an immediate and significant induction of dystonia over the first hour of administration (AUC_{-1-0h}) when compared to vehicle-A (Fig.6.2 F). This resulted overall in a significant increase in total (AUC_{0-5h}), on-time and peak scores when compared to vehicle-A/B-treated animals over the 5 hours after the L-DOPA/vehicle-B administration (Fig. 6.2 B, C & E).

Pre-treatment with scopolamine (0.1 & 0.3 mg/kg) potentiated the duration of L-DOPA-induced dystonia resulting in a significant increase in on-time (0.3 mg/kg) and on-time > 2 scores when compared to L-DOPA alone (Fig. 6.2 C & D), although there was no effect on total (AUC_{0-5h}) and peak score (Fig. 6.2 B & E).

Induction of dystonia by the L-DOPA/scopolamine combination remained significantly different to scopolamine alone treatment as measured by total (AUC_{0-5h}), on-time (0.3 mg/kg), on-time > 2 or peak score (Fig. 6.2 C – E).

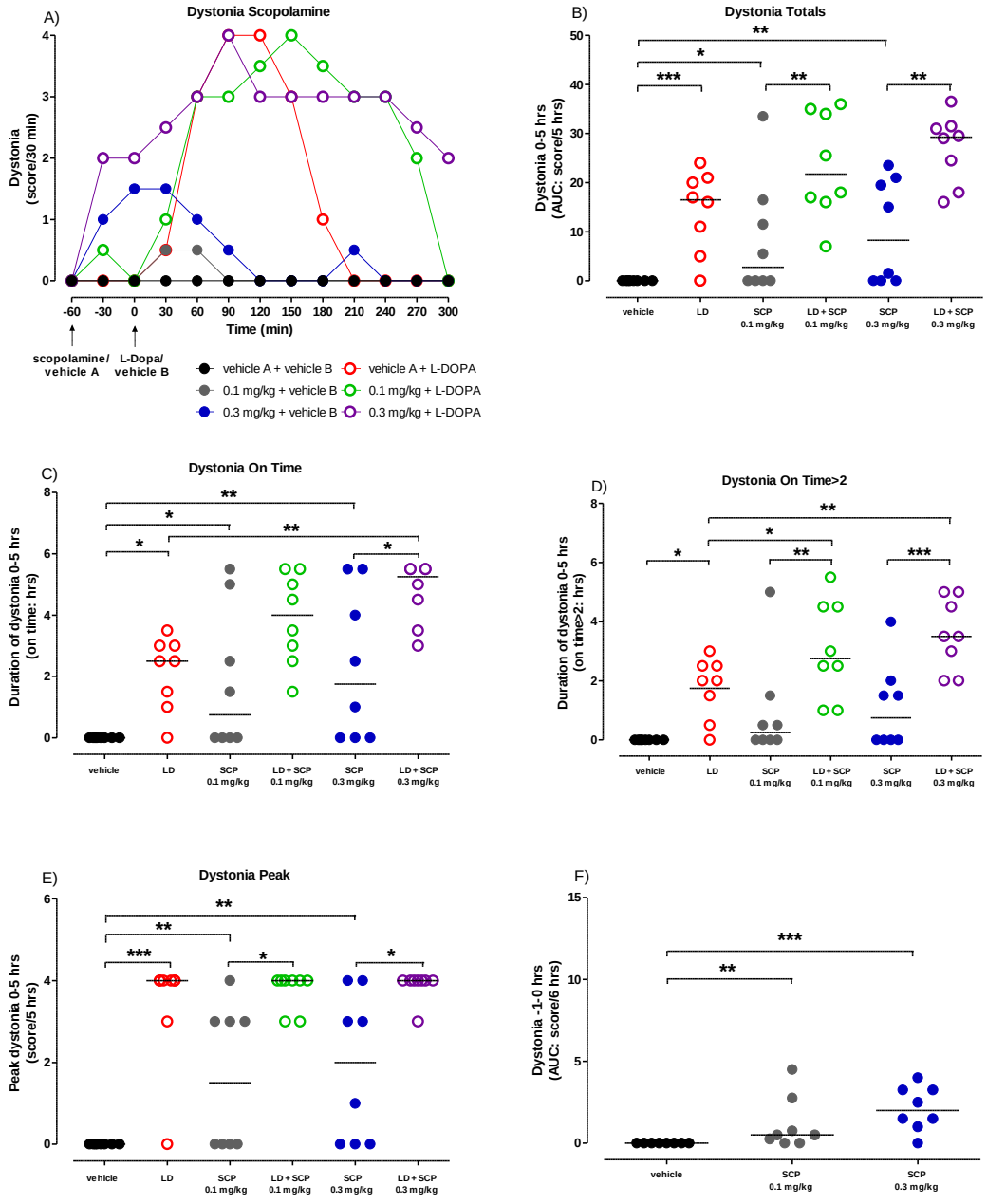


Figure 6.2 Effect of scopolamine on L-DOPA-induced dystonia in MPTP-treated common marmosets

Scopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Dystonia time course and B) Total dystonia ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak dystonia after L-DOPA/vehicle-B administration; F) Total dystonia in the first hour after scopolamine/vehicle ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=13.54$; Df (5,47); $p<0.0001$; C) $F=9.885$; Df (5,47); $p<0.0001$; D) $F=11.04$; Df (5,47); $p<0.0001$; E) $F=12.26$; Df (5,47); $p<0.0001$; F) $F=16.58$; Df (2,23); $p=0.0002$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.1.1.3 Chorea

Administration of scopolamine alone (0.1 & 0.3 mg/kg) resulted in a significant induction of chorea over first hour of administration (AUC_{-1-0h}), when compared to vehicle-A treatment (Fig. 6.3 F), and overall producing a significant effect in total (AUC_{0-5h}), on-time and peak over 5 hrs after L-DOPA/vehicle-B administration (Fig. 6.3 B, C & E).

Pre-treatment with scopolamine (0.1 & 0.3 mg/kg) significantly increased total (AUC_{0-5h}) and extended duration of L-DOPA-induced chorea as measured by on-time and on-time > 2 scores when compared to that produced by L-DOPA alone (Fig. 6.3 C & D), although there was no significant effect on peak scores (Fig. 6.3 B & F).

When compared to scopolamine alone (0.1 & 0.3 mg/kg), the combined L-DOPA/scopolamine treatment resulted in a significant increase in chorea as measured by total (AUC_{0-5h}), on-time, on-time > 2 and peak scores (0.1 mg/kg) (Fig. 6.3 B – E).

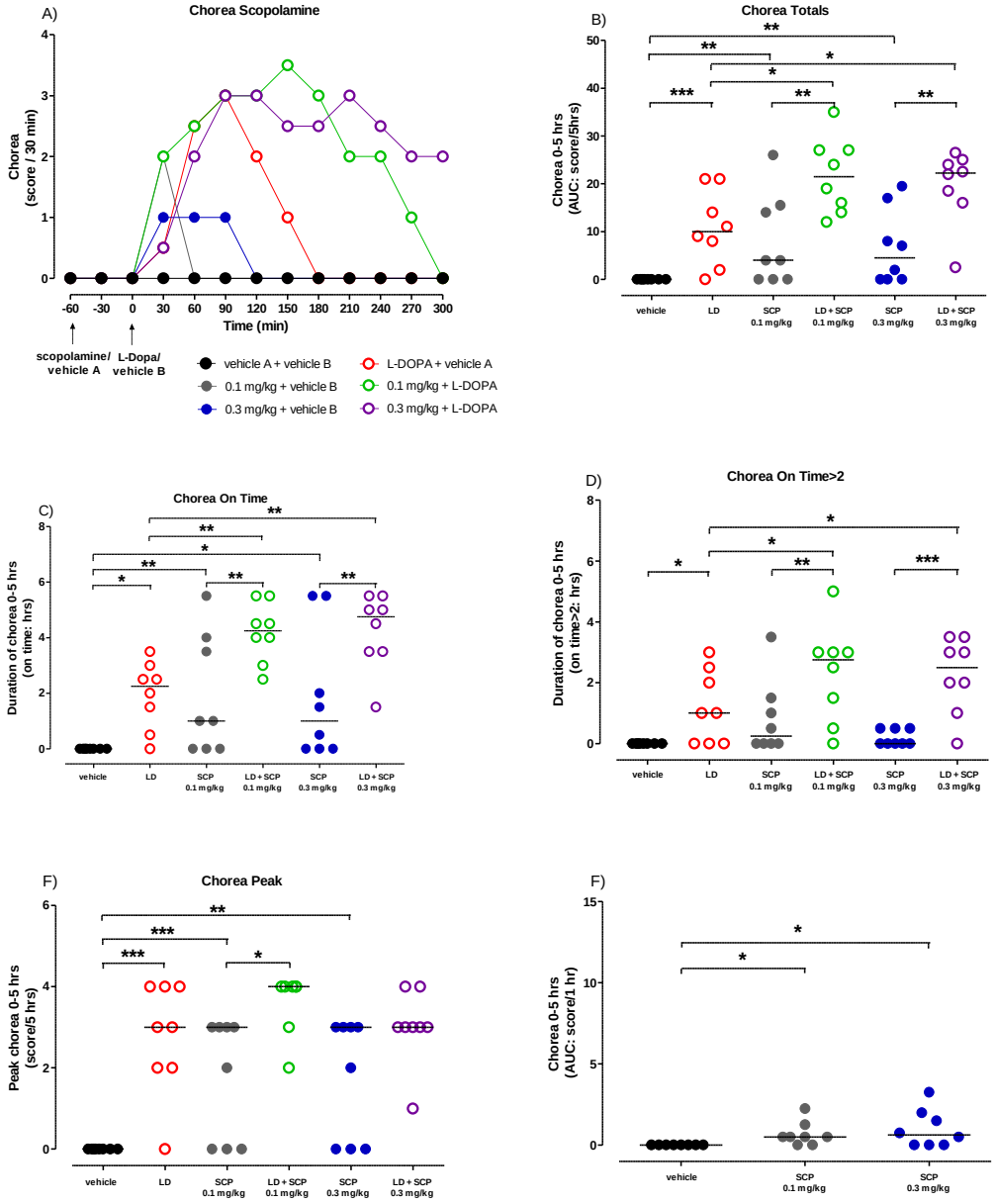


Figure 6.3 Effect of scopolamine on L-DOPA-induced chorea in MPTP-treated common marmosets

Scopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Chorea time course and B) Total chorea ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak chorea after L-DOPA/vehicle-B administration; F) Total chorea in the first hour after scopolamine/vehicle ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=16.12$; Df (5,47); $p<0.0001$; C) $F=11.19$; Df (5,47); $p<0.0001$; D) $F=10.22$; Df (5,47); $p<0.0001$; E) $F=11.96$; Df (5,47); $p<0.0001$; F) $F=5.471$; Df (2,23); $p=0.0176$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.1.2 Peripherally acting methylscopolamine

6.3.1.2.1 Dyskinesia

Methylscopolamine alone (0.1 & 0.3 mg/kg) had no effect on induction of dyskinesia within the first hour of administration (AUC_{-1-0h}) or throughout the 5 hours after L-DOPA/vehicle-B administration when compared to vehicle treatment (Fig. 6.4 B – F).

Unexpectedly, pre-treatment with methylscopolamine (0.1 & 0.3 mg/kg) significantly reduced L-DOPA-induced dyskinesia as measured by total (AUC_{0-5h}), on-time and peak scores when compared to L-DOPA alone (Fig. 6.4 B, C, E), although when compared to methylscopolamine alone (0.1 & 0.3 mg/kg), dyskinesia following combined L-DOPA/methylscopolamine treatment remained significantly increased as measured by totals (AUC_{0-5h}), on-time, on-time > 2 and peak (Fig. 6.4 B – E).

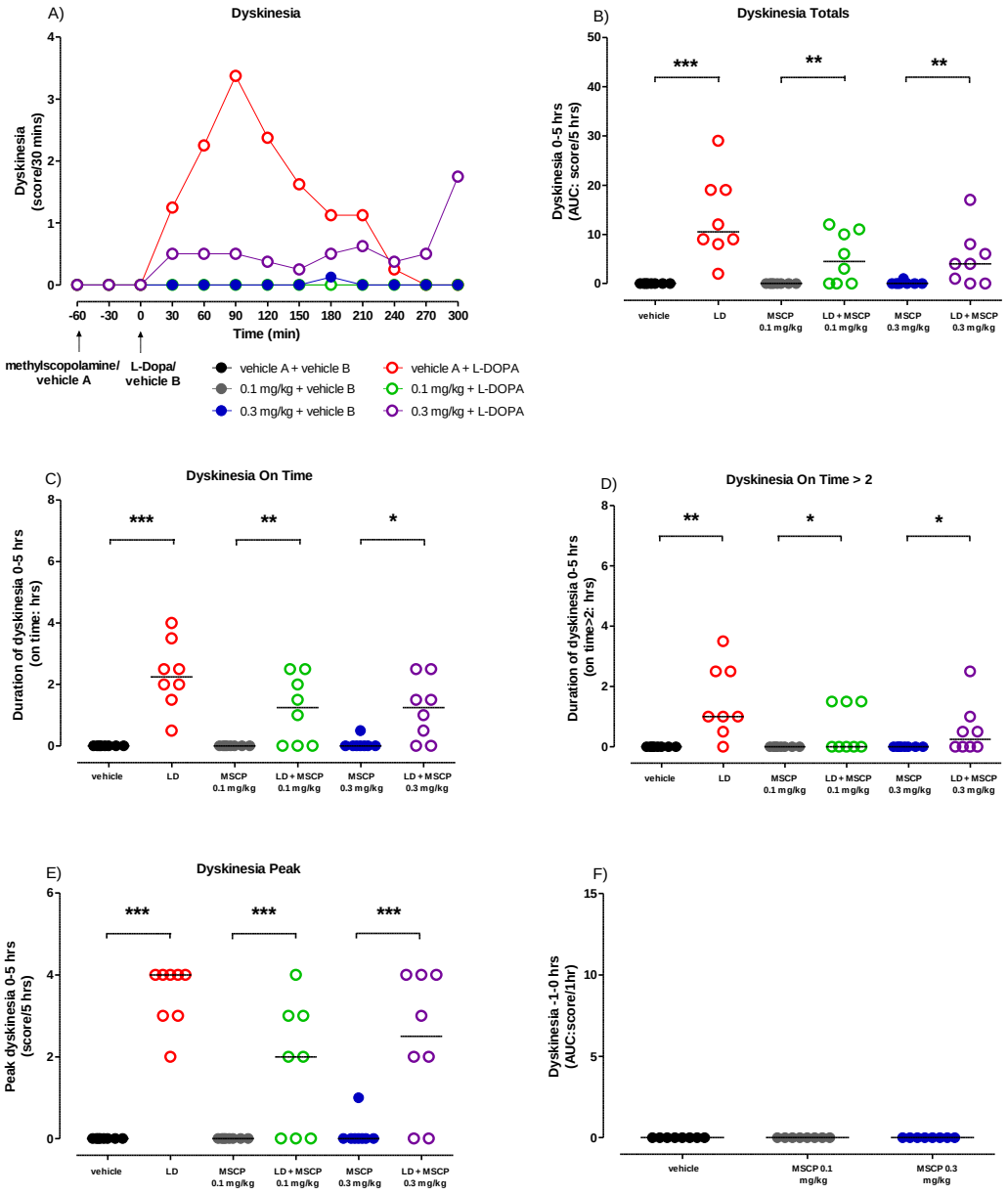


Figure 6.4 Effect of methylscopolamine on L-DOPA-induced dyskinesia in MPTP-treated common marmosets

A) Dyskinesia time course and B) Total dyskinesia ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak dyskinesia after L-DOPA/vehicle-B administration; F) Total dyskinesia in the first hour after methylscopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=15.55$; Df (5,47); $p<0.0001$; C) $F=12.22$; Df (5,47); $p<0.0001$; D) $F=5.590$; Df (5,47); $p=0.0007$; E) $F=19.34$; Df (5,47); $p<0.0001$; F) $F=1.000$; Df (2,23); $p=0.3927$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.1.2.2 Dystonia

Methylscopolamine alone (0.1 & 0.3 mg/kg) had no effect on induction of dystonia within the first hour of administration (AUC_{-1-0h}) and throughout the 5 hours after L-DOPA/vehicle-B administration when compared to vehicle treatment (Fig. 6.5 B – F).

As seen with dyskinesia, pre-treatment with methylscopolamine significantly reduced L-DOPA-induced dystonia as measured by total (AUC_{0-5h}), on-time, on-time > 2 and peak (0.1 mg/kg) when compared to L-DOPA alone (Fig. 6.5 B – E), although when compared to methylscopolamine alone (0.1 & 0.3 mg/kg) following combined L-DOPA/methylscopolamine treatment dystonia remained significantly increased as measured by total (AUC_{0-5h}), on-time (0.3 mg/kg) and peak scores (Fig. 6.5 B, C & E).

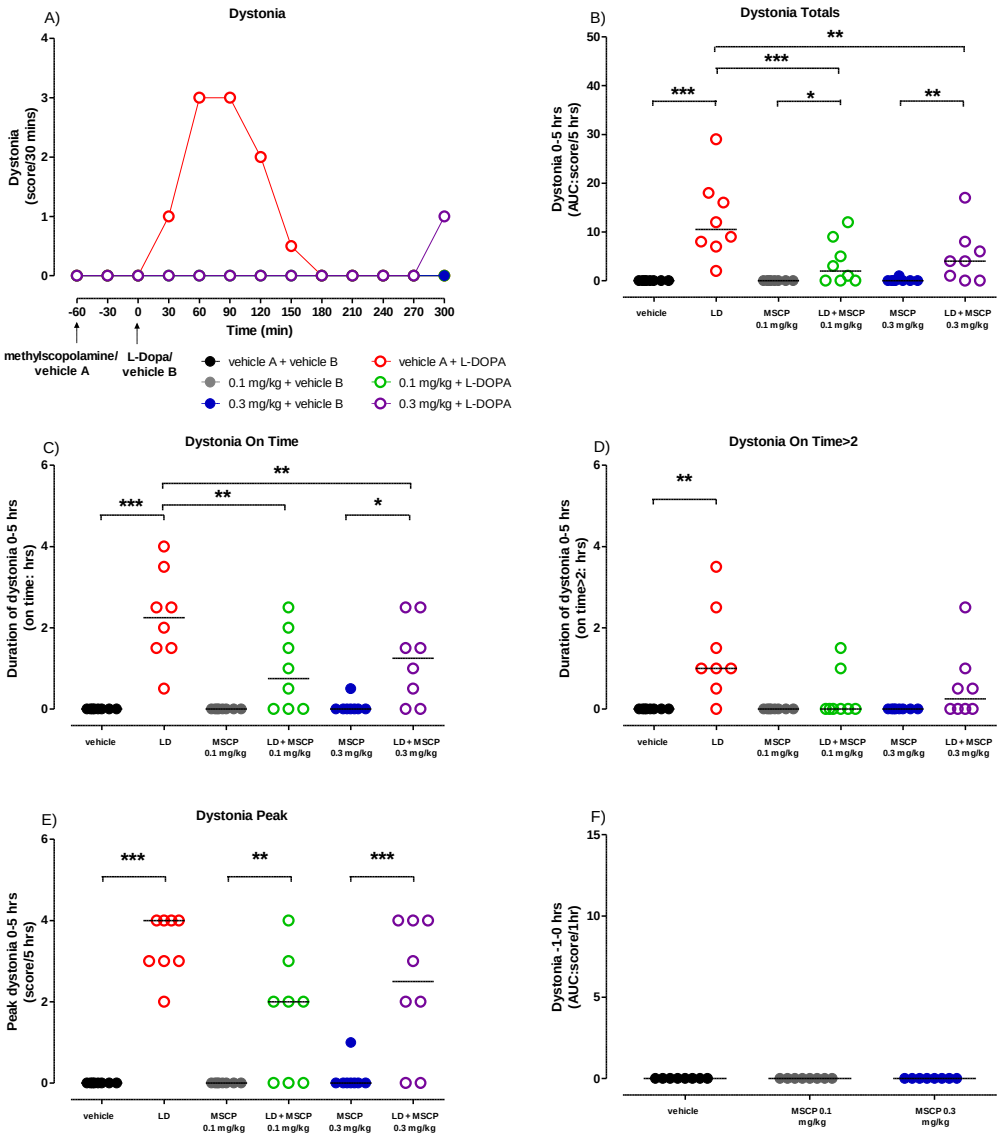


Figure 6.5 Effect of methylscopolamine on L-DOPA-induced dystonia in MPTP-treated common marmosets

Methylscopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Dystonia time course and B) Total dystonia ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak dystonia after L-DOPA/vehicle-B administration; F) Total dystonia in the first hour after methylscopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=15.86$; Df (5,47); $p<0.0001$; C) $F=12.20$; Df (5,47); $p<0.0001$; D) $F=5.650$; Df (5,47); $p=0.0006$; E) $F=19.03$; Df (5,47); $p<0.0001$; F) $F=0.8884$; Df (2,23); $p=0.4333$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.1.2.3 Chorea

Methylscopolamine alone (0.1 & 0.3 mg/kg) had no effect on induction of chorea within the first hour of administration (AUC_{-1-0h}) and throughout the 5 hours after L-DOPA/vehicle-B administration when compared to vehicle treatment (Fig. 6.6 B – F).

As seen with dyskinesia and dystonia, pre-treatment with methylscopolamine (0.1 & 0.3 mg/kg) significantly reduced L-DOPA-induced chorea as measured by total (AUC_{0-5h}), on-time, on-time > 2 and peak scores when compared to L-DOPA alone (Fig. 6.6 B – E).

When compared to methylscopolamine alone (0.1 & 0.3 mg/kg) the combined L-DOPA/methylscopolamine treatment remained significantly increased as measured by total (AUC_{0-5h}), on-time (0.3 mg/kg) and peak scores (Fig. 6.6 B, C & E).

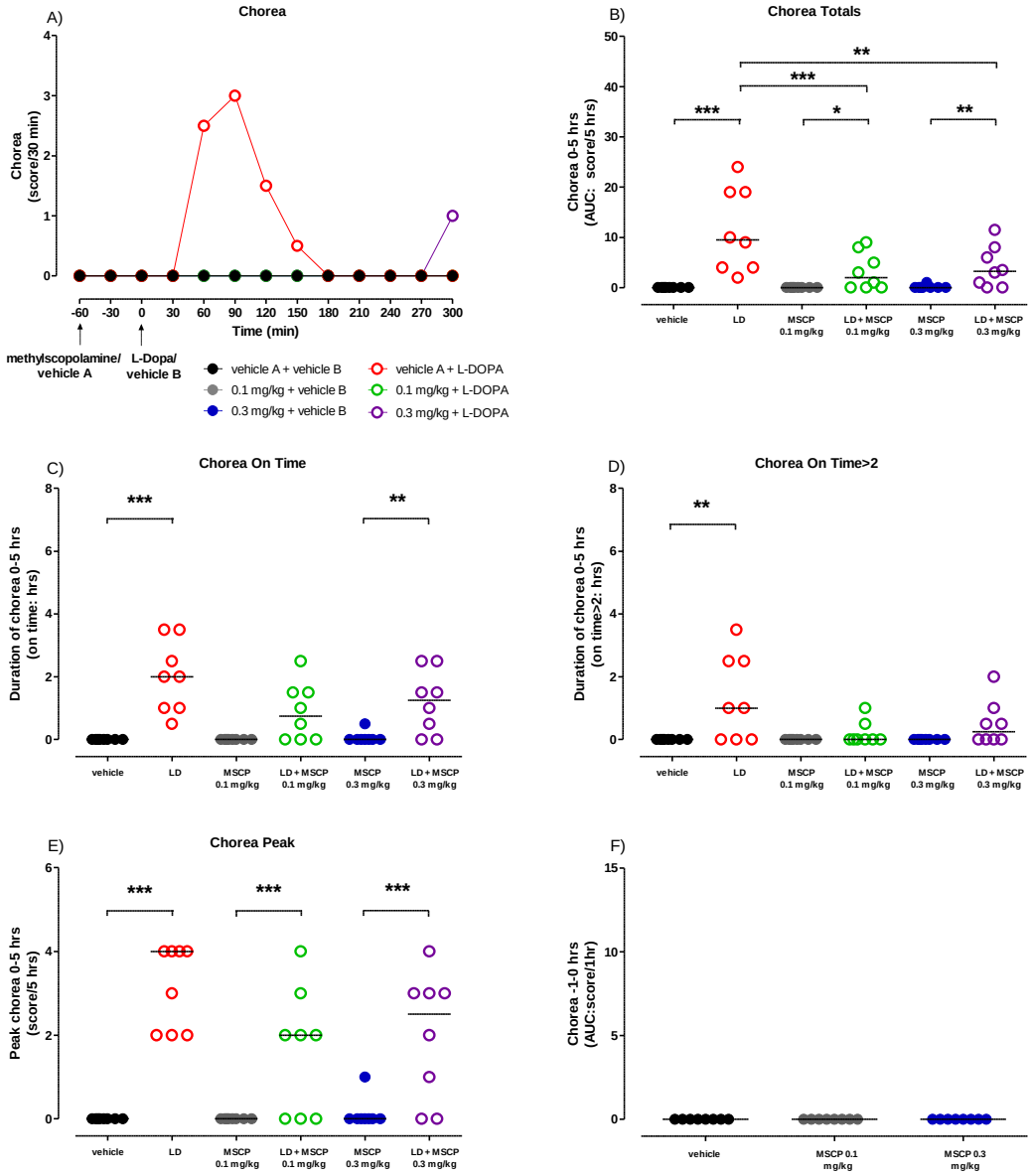


Figure 6.6 Effect of methylscopolamine on L-DOPA-induced chorea in MPTP-treated common marmosets

Methylscopolamine (0.1 & 0.3 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=8) A) Chorea time course and B) Total chorea ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak chorea after L-DOPA/vehicle-B administration; F) Total chorea in the first hour after methylscopolamine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=16.40$; Df (5,47); $p<0.0001$; C) $F=10.65$; Df (5,47); $p<0.0001$; D) $F=5.056$; Df (5,47); $p=0.0014$; E) $F=18.84$; Df (5,47); $p<0.0001$; F) $F=1.000$; Df (2,23); $p=0.3927$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.2 Clinically used selective M1 anticholinergics

6.3.2.1 Trihexyphenidyl

6.3.2.1.1 Dyskinesia

Trihexyphenidyl alone (0.5 & 1 mg/kg) produced an immediate and significant induction of dyskinesia over the first hour of treatment (AUC_{-1-0h}) when compared to vehicle-A (Fig. 6.7 F). This resulted overall in a significant increase in total (AUC_{0-5h}), on-time and peak scores after L-DOPA/vehicle-B administration when compared to vehicle-A/B treatment (Fig. 6.7 B, C & E).

Pre-treatment with trihexyphenidyl (0.5 & 1 mg/kg) significantly increased the duration of L-DOPA-induced dyskinesia as measured by total (AUC_{0-5h}), on-time and on-time > 2 (1 mg/kg) when compared to L-DOPA alone (Fig. 6.7 B, C & D), although there was no significant effect of trihexyphenidyl on peak scores (Fig. 6.1 E). When compared to trihexyphenidyl alone (0.5 & 1 mg/kg) the combination L-DOPA/trihexyphenidyl remained significantly greater as measured by totals (AUC_{0-5h}), on-time, on-time > 2 and peak (0.5 mg/kg) (Fig. 6.7 B – E).

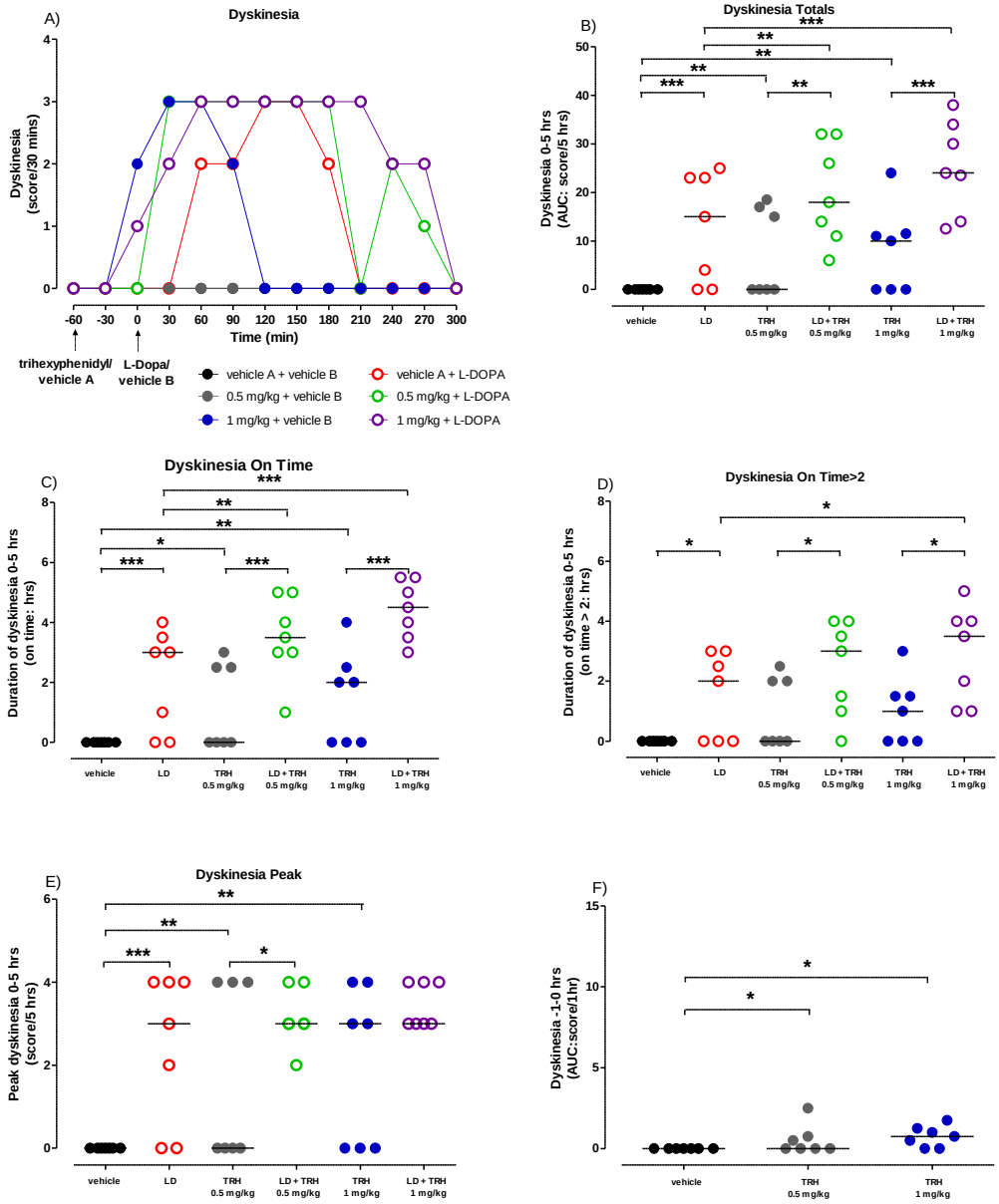


Figure 6.7 Effect of trihexyphenidyl on L-DOPA-induced dyskinesia in MPTP-treated common marmosets

Trihexyphenidyl (0.5 & 1 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Dyskinesia time course and B) Total dyskinesia ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak dyskinesia after L-DOPA/vehicle-B administration; F) Total dyskinesia in the first hour after trihexyphenidyl/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=17.69$; Df (5,41); $p<0.0001$; C) $F=27.03$; Df (5,41); $p<0.0001$; D) $F=9.915$; Df (5,41); $p<0.0001$; E) $F=9.882$; Df (5,41); $p<0.0001$; F) $F=6.149$; Df (5,41); $p=0.0145$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.2.1.2 Dystonia

Administration of trihexyphenidyl alone (0.5 & 1 mg/kg) produced an immediate and significant induction of dystonia over the first hour of administration (AUC_{-1-0h}) when compared to vehicle-A-treated animals (Fig. 6.8 F). This resulted in an increase in total (AUC_{0-5h}) scores and extended duration of dystonia as measured by on-time, which was significantly different to vehicle-A/B treatment (Fig. 6.8 B & C).

Pre-treatment with trihexyphenidyl (0.5 & 1 mg/kg) had no effect on peak scores but significantly increased total (AUC_{0-5h}) scores (1 mg/kg) and duration of L-DOPA-induced dystonia when compared to L-DOPA alone (Fig. 6.8 B, C & E). When compared to trihexyphenidyl alone, the combination L-DOPA/trihexyphenidyl remained significantly different to trihexyphenidyl alone as measured by total (AUC_{0-5h}), on-time, on-time > 2 (1 mg/kg) and peak scores (Fig. 6.8 B – E).

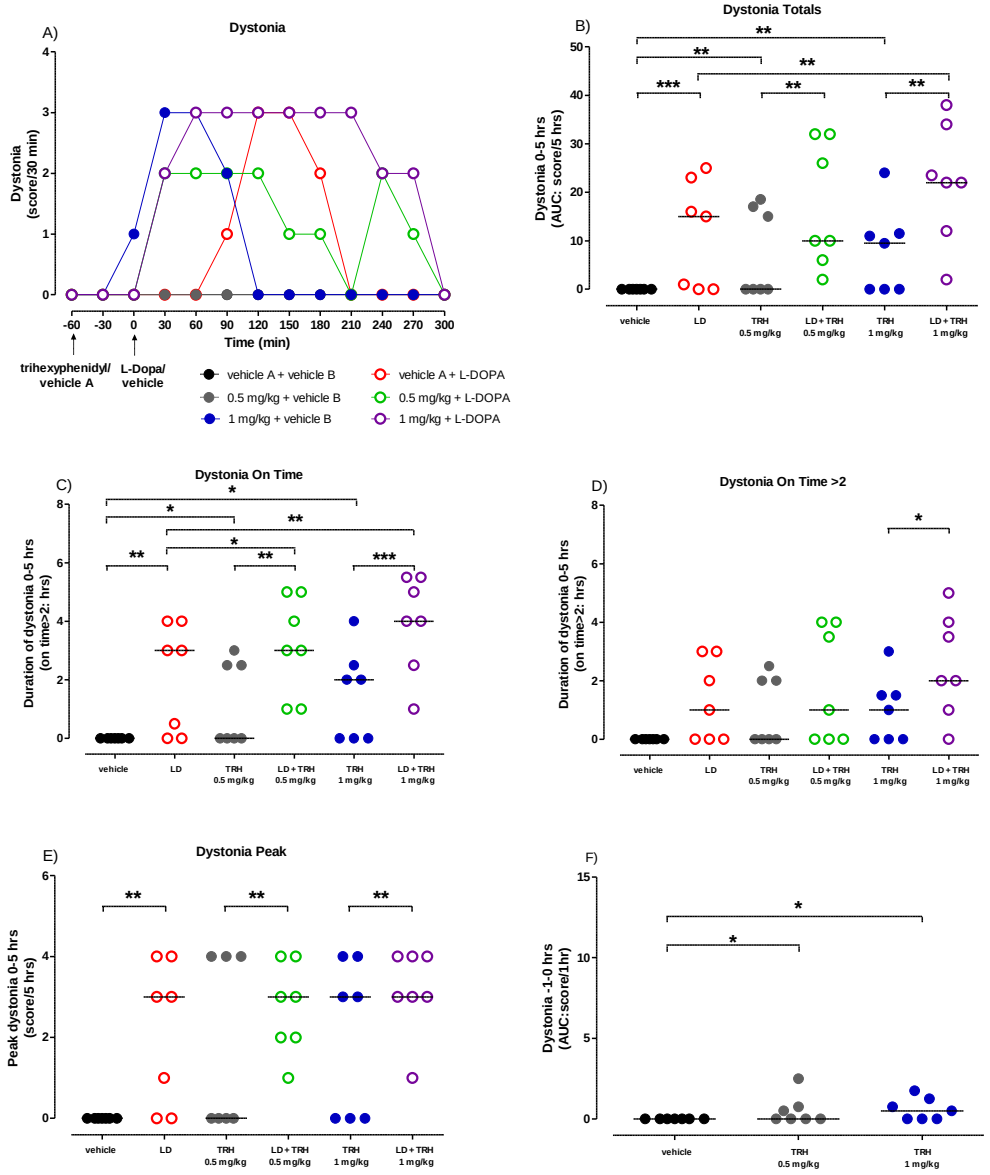


Figure 6.8 Effect of trihexyphenidyl on L-DOPA-induced dystonia in MPTP-treated common marmosets

Trihexyphenidyl (0.5 & 1 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Dystonia time course and B) Total dystonia ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak dystonia after L-DOPA/vehicle-B administration; F) Total dystonia in the first hour after trihexyphenidyl/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=14.79$; Df (5,41); $p<0.0001$; C) $F=16.88$; Df (5,41); $p<0.0001$; D) $F=5.822$; Df (5,41); $p=0.0007$; E) $F=9.524$; Df (5,41); $p<0.0001$; F) $F=4.548$; Df (5,41); $p=0.0339$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$.

6.3.2.1.3 Chorea

Trihexyphenidyl alone (0.5 & 1 mg/kg) had no effect of induction of chorea over the first hour of administration (AUC_{-1-0h}) when compared to vehicle-A treatment (Fig. 6.9 F), however, produced a significant effect in total (AUC_{0-5h}), on-time and peak over 5 hrs after L-DOPA/vehicle-B administration when compared to vehicle-A/B treatment (Fig. 6.9 B, C & E).

Pre-treatment with trihexyphenidyl (0.5 & 1 mg/kg) significantly increased totals (AUC_{0-5h}), extended duration of L-DOPA-induced chorea as measured by on-time and increased the peak (1 mg/kg) when compared to L-DOPA alone (Fig. 6.9 B. C & E). When compared to trihexyphenidyl alone (0.5 & 1 mg/kg), the combined L-DOPA/trihexyphenidyl treatment remained significantly different as measured by total (AUC_{0-5h}) and peak scores, on-time and on-time > 2 (1 mg/kg) (Fig. 6.9 B – E).

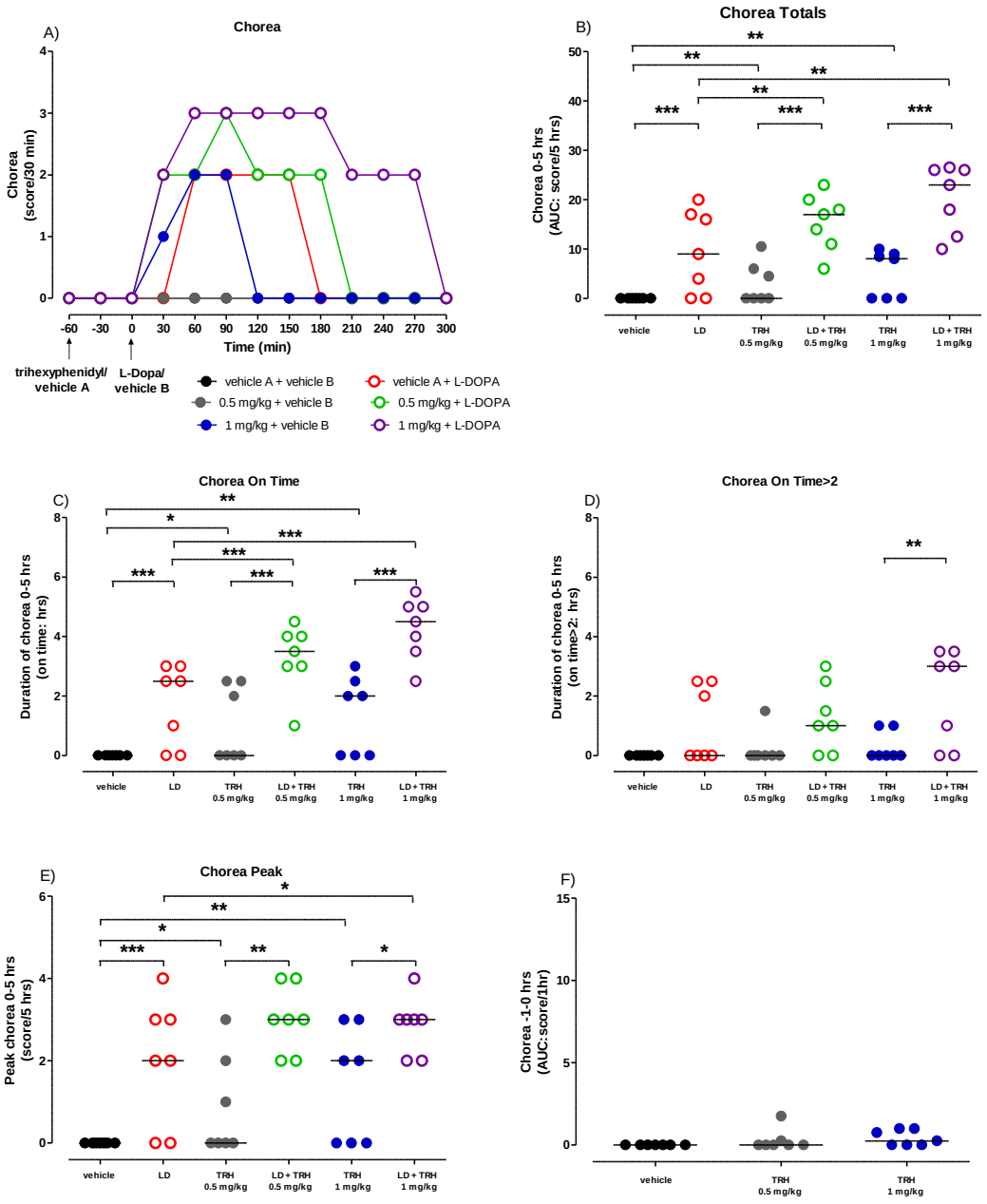


Figure 6.9 Effect of trihexyphenidyl on L-DOPA-induced chorea in MPTP-treated common marmosets

Trihexyphenidyl (0.5 & 1 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Chorea time course and B) Total chorea ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak chorea after L-DOPA/vehicle-B administration; F) Total chorea in the first hour after trihexyphenidyl/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=23.88$; Df (5,41); $p<0.0001$; C) $F=34.20$; Df (5,41); $p<0.0001$; D) $F=6.534$; Df (5,41); $p=0.0003$; E) $F=12.20$; Df (5,41); $p<0.0001$; F) $F=2.967$; Df (5,41); $p=0.0897$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.2.2 Benztropine

6.3.2.2.1 Dyskinesia

Benztropine alone (0.25 & 0.5 mg/kg) immediately and significantly induced dyskinesia over the first hour of administration (AUC_{-1-0h}) when compared to vehicle-A treatment (Fig. 6.10 F). This resulted overall in a significant increase in total (AUC_{0-5h}), on-time and peak over the 5 hours after L-DOPA/vehicle-B administration when compared to vehicle-A/B treatment (Fig. 6.10 C & E).

Pre-treatment with benztropine (0.25 & 0.5 mg/kg) had no effect on L-DOPA-induced dyskinesia when compared to L-DOPA alone (Fig. 6.10 B – E), however, when compared to benztropine alone, the combination L-DOPA/benztropine treatment resulted in a significant increase in total (AUC_{0-5h}) scores and on-time at the lower dose (0.25 mg/kg) (Fig. 6.10 B & C).

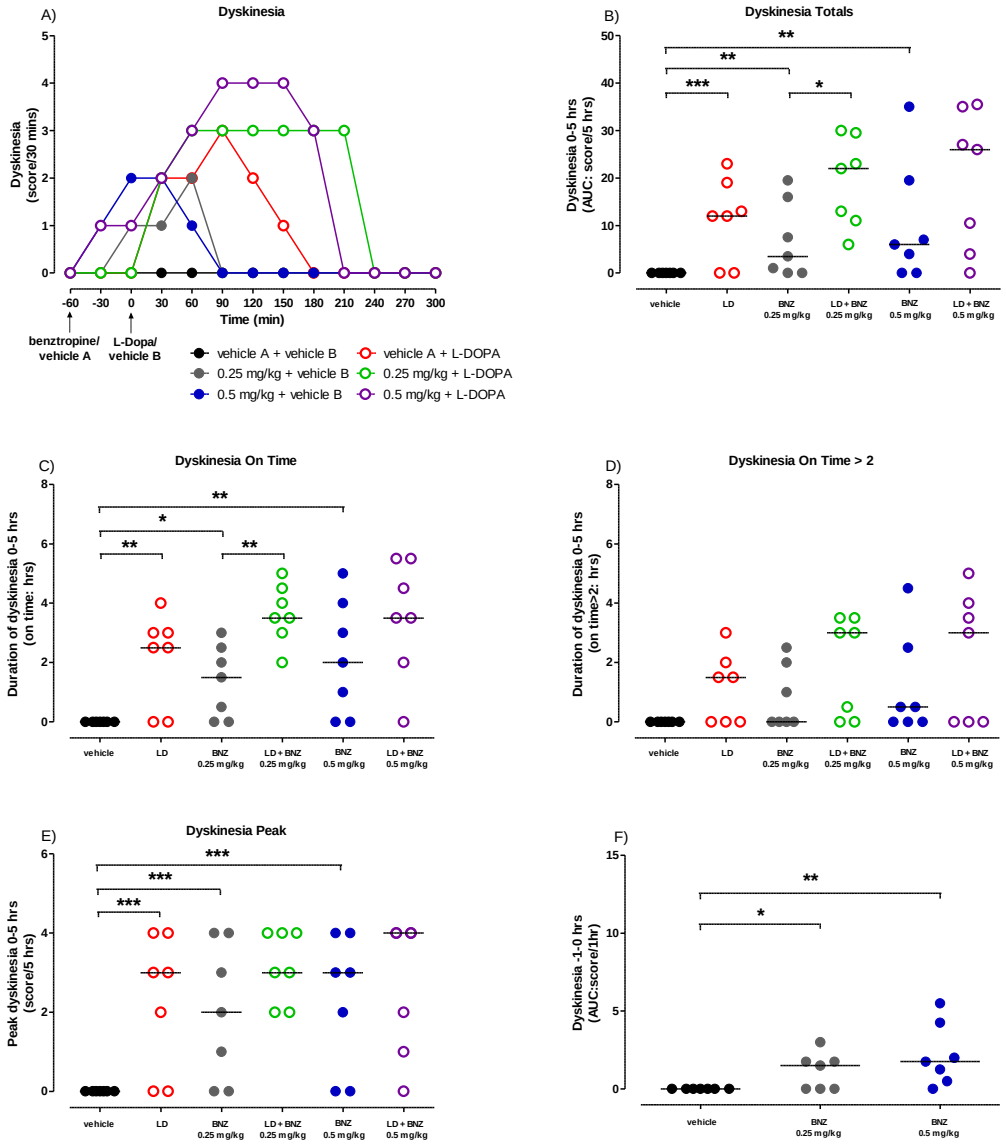


Figure 6.10 Effect of benztropine on L-DOPA-dyskinesia in MPTP-treated common marmosets

Benztropine (0.25 & 0.5 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Dyskinesia time course and B) Total dyskinesia ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak dyskinesia after L-DOPA/vehicle-B administration; F) Total dyskinesia in the first hour after benztropine/ vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=10.81$; Df (5,41); $p<0.0001$; C) $F=11.13$; Df (5,41); $p<0.0001$; D) $F=3.845$ Df (5,41); $p=0.0082$; E) $F=10.10$; Df (5,41); $p<0.0001$; F) $F=11.93$; Df (5,41); $p=0.0014$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.2.2.2 Dystonia

Administration of benztropine alone (0.25 & 0.5 mg/kg) produced an immediate and significant induction of dystonia over the first hour (AUC_{-1-0h}) when compared to vehicle-A-treated animals (Fig. 6.11 F). This resulted in an extended duration of dystonia as measured by on-time and peak at the higher dose (0.5 mg/kg) over the 5 hrs, which was significantly different to vehicle-A/B treatment (Fig. 6.11 C & E).

Pre-treatment with benztropine (0.25 & 0.5 mg/kg) had no effect on L-DOPA-induced dystonia when compared to L-DOPA treatment (Fig. 6.11 B – E). There was no effect of the combination L-DOPA/benztropine treatment when compared to benztropine alone as measured by total (AUC_{0-5h}), scores on-time, on-time > 2 or peak (Fig. 6.11 B – E).

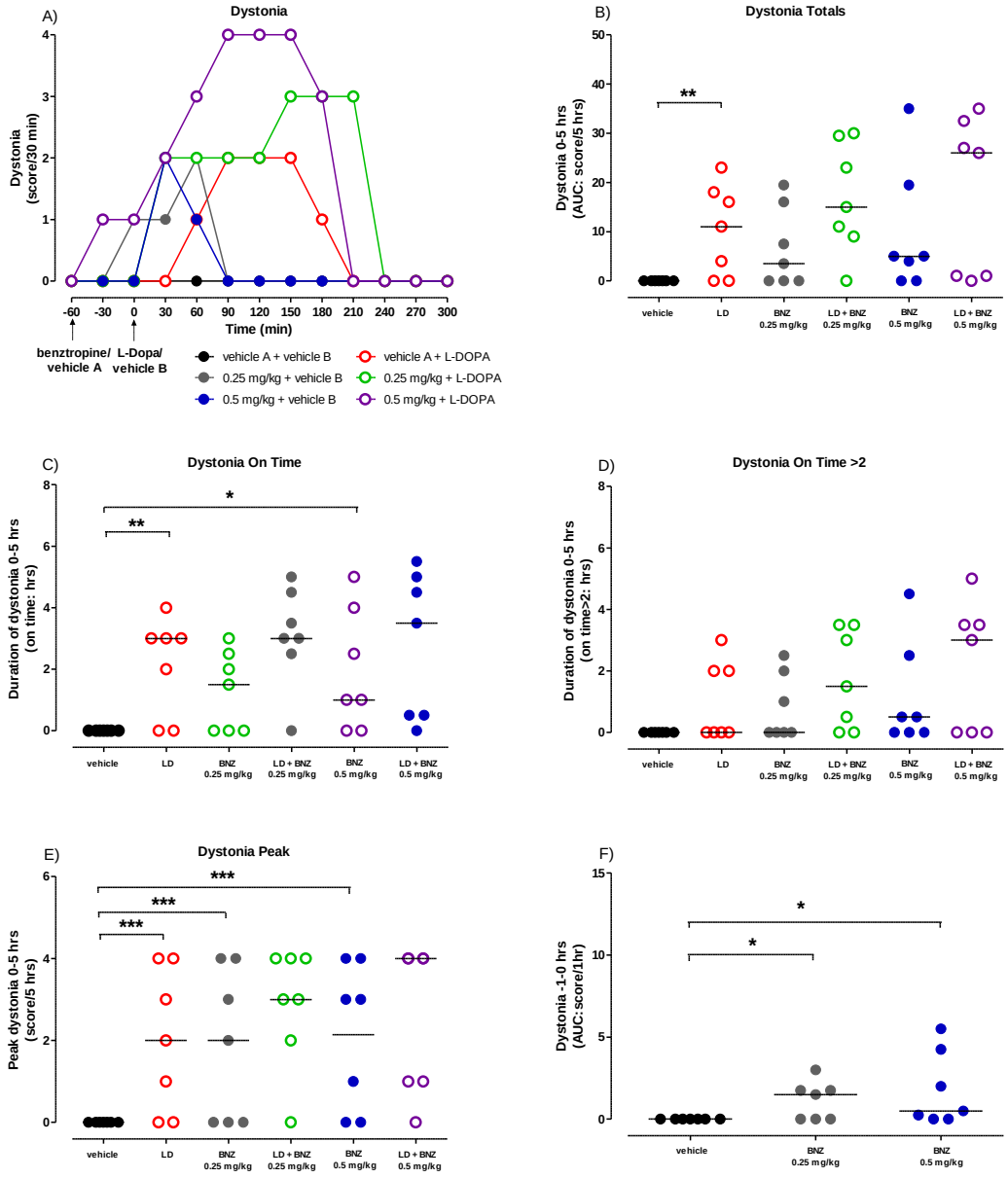


Figure 6.11 Effect of benztropine on L-DOPA-induced dystonia in MPTP-treated common marmosets

Benztropine (0.25 & 0.5 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Dystonia time course and B) Total dystonia ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak dystonia after L-DOPA/vehicle-B administration; F) Total dystonia in the first hour after benztropine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=7.542$; Df (5,41); $p=0.0001$; C) $F=6.237$; Df (5,41); $p=0.0004$; D) $F=3.913$; Df (5,41); $p=0.0075$; E) $F=9.390$; Df (5,41)$p<0.0001$; F) $F=6.681$; Df (5,41); $p=0.0112$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.2.2.3 Chorea

Benztropine alone (0.25 & 0.5 mg/kg) produced immediate and significant induction of chorea over the first hour (AUC_{-1-0h}) when compared to vehicle-A-treated animals (Fig. 6.12 F). This produced a significant effect in total (AUC_{0-5h}) and peak scores, and on-time over the 5 hrs when compared to vehicle-A/B treatment (Fig. 6.12 B, C & F).

Pre-treatment with benztropine (0.25 & 0.5 mg/kg) significantly extended the duration of L-DOPA-induced chorea when compared to L-DOPA alone (Fig. 6.12 C). When compared to benztropine alone (0.25 & 0.5 mg/kg), the combined L-DOPA/benztropine treatment remained significantly different as measured by total (AUC_{0-5h}) scores and on-time at the higher dose (0.5 mg/kg) (Fig. 6.12 B & C).

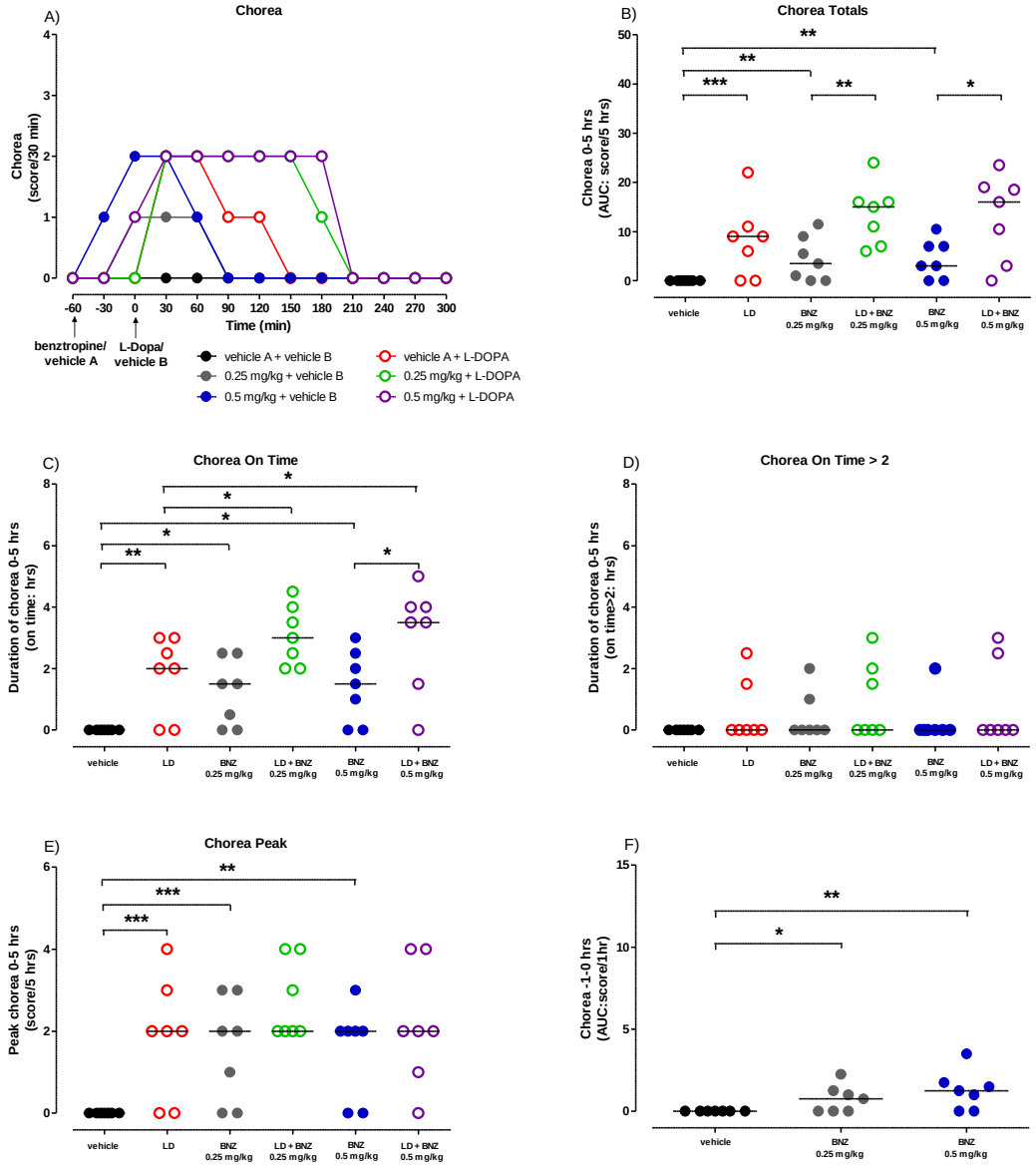


Figure 6.12 Effect of benztropine on L-DOPA-induced chorea in MPTP-treated common marmosets

Benztropine (0.25 & 0.5 mg/kg s.c.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=7) A) Chorea time course and B) Total chorea ($AUC_{0\text{-}5h}$), C) On-time, D) On-time > 2 and E) Peak chorea after L-DOPA/vehicle-B administration; F) Total chorea in the first hour after benztropine/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=11.36$; Df (5,41); $p<0.0001$; C) $F=11.72$; Df (5,41); $p<0.0001$; D) $F=1.063$; Df (5,41); $p=0.4003$; E) $F=8.756$; Df (5,41); $p<0.0001$; F) $F=8.670$; Df (5,41); $p=0.0047$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.3 Selective M4 antagonist - NBI-675

6.3.3.1 Dyskinesia

NBI-675 alone (1 – 7.5 mg/kg) had no effect on induction of dyskinesia over the first hour (AUC_{-1-0h}) and over the 5 hours ($AUC_{0-5.5h}$) after the L-DOPA/vehicle-B administration when compared to vehicle-A/B treatment (Fig. 6.13 B – F).

Pre-treatment with NBI-675 (1 – 7.5 mg/kg) had no effect on L-DOPA-induced dyskinesia when compared to L-DOPA alone (Fig. 6.13 B – E), however, when compared to NBI-675 alone, the combination L-DOPA/NBI-675 treatment remained significantly different as measured by total scores ($AUC_{0-5.5h}$) (1 & 5 mg/kg), on-time (5 mg/kg) and peak scores (Fig. 6.13 B, C & E).

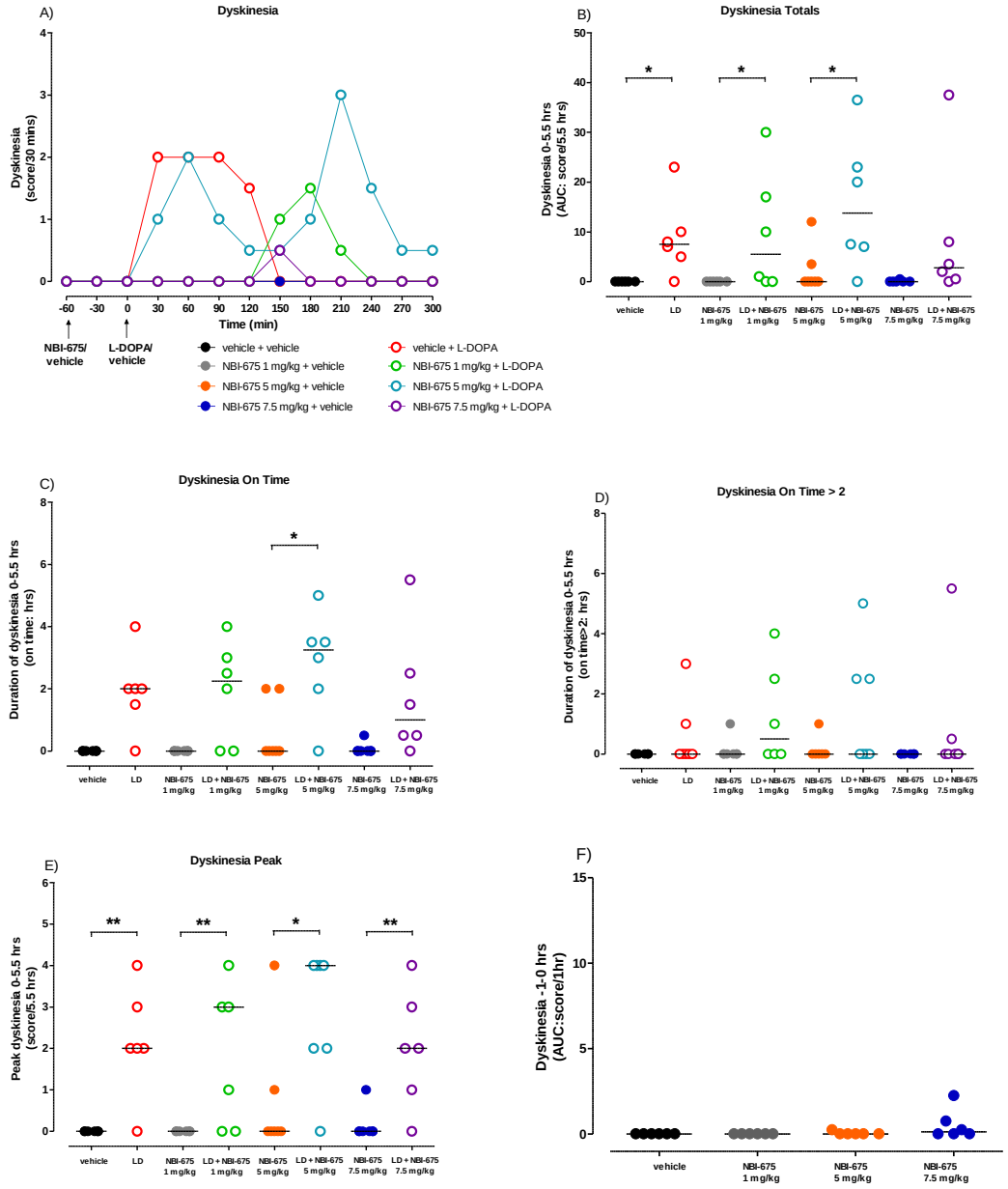


Figure 6.13 Effect of NBI-675 on L-DOPA-induced dyskinesia in MPTP-treated common marmosets

NBI-675 (1 – 7.5 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=6) A) Dyskinesia time course and B) Total dyskinesia ($AUC_{0\text{-}5.5h}$); C) On-time, D) On-time > 2 and E) Peak dyskinesia after L-DOPA/vehicle-B administration; F) Total dyskinesia in the first hour after NBI-675/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=6.513$; Df (7,47); $p<0.0001$; C) $F=6.183$; Df (7,47); $p<0.0001$; D) $F=1.546$; Df (7,47); $p=0.1843$; E) $F=8.016$; Df (7,47); $p<0.0001$; F) $F=2.783$; Df (3,23); $p=0.0770$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.3.3.2 Dystonia

NBI-675 alone (1 – 7.5 mg/kg) had no effect on induction of dystonia over the first hour (AUC_{-1-0h}) when compared to vehicle-A (Fig. 6.14 F) and no effect over the 5 hrs ($AUC_{0-5.5h}$) after the L-DOPA/vehicle-B administration when compared to vehicle-A/B treatment (Fig. 6.14 B – E).

Pre-treatment with NBI-675 (1 – 7.5 mg/kg) had no significant effect on L-DOPA-induced dystonia (Fig. 6.14 B – E), however, when compared to NBI-675 alone, the combination L-DOPA/NBI-675 treatment remained significantly different as measured by total ($AUC_{0-5.5h}$) (5 mg/kg) and on-time at the middle dose (5 mg/kg), while peak tended to be increased (Fig. 6.14 B, C & E).

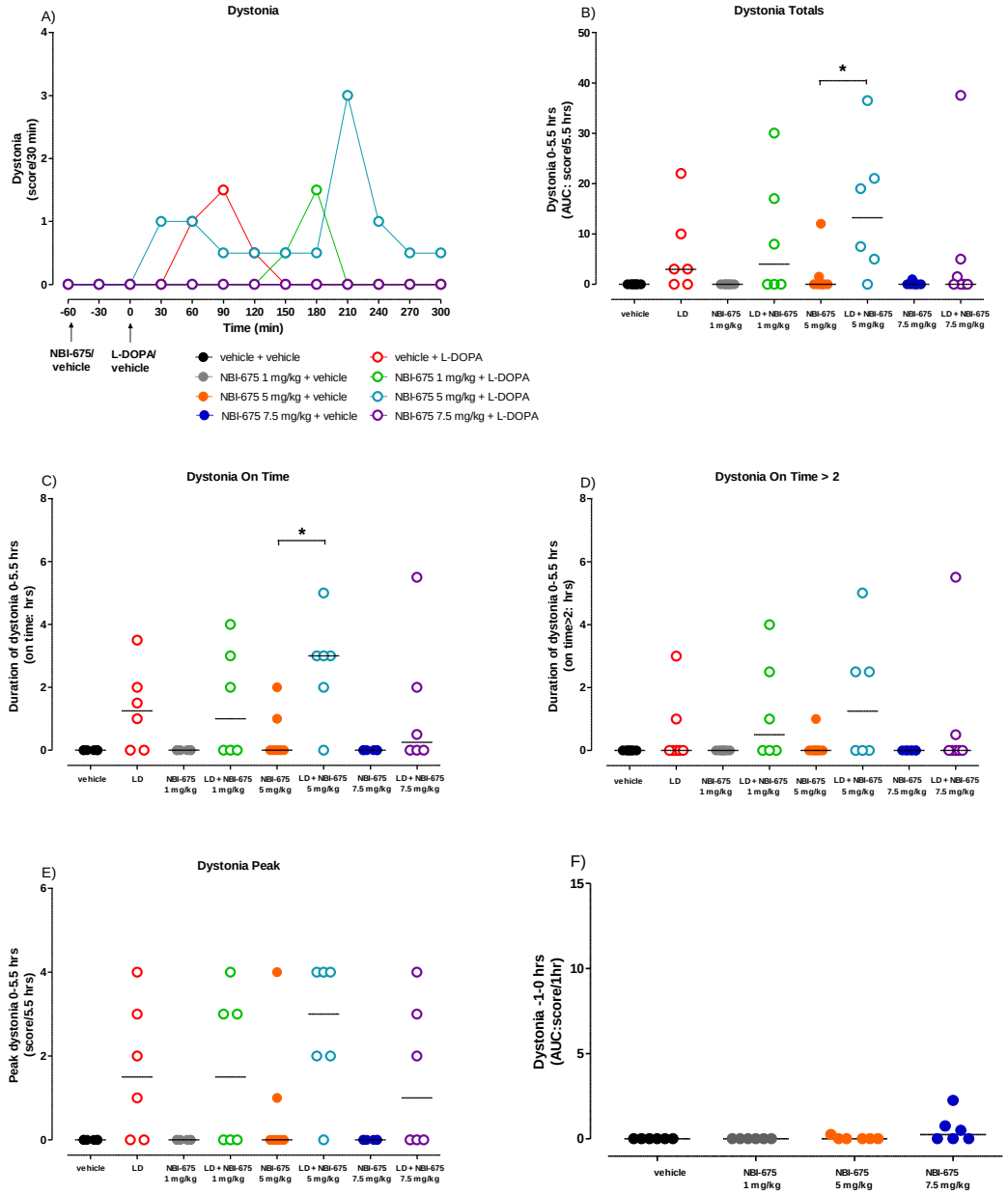


Figure 6.14 Effect of NBI-675 on L-DOPA-induced dystonia in MPTP-treated common marmosets

NBI-675 (1 – 7.5 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=6) A) Dystonia time course and B) Total dystonia ($AUC_{0\text{-}5.5h}$), C) On-time, D) On-time > 2 and E) Peak dystonia after L-DOPA/vehicle-B administration; F) Total dystonia in the first hour after NBI-675/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) F=4.604; Df (7,47); p=0.0010; C) F=4.685; Df (7,47); p=0.0009; D) F=2.175; Df (7,47); p=0.0608; E) F=5.073; Df (7,47); p=0.0005; F) F=3.143; Df (3,23); p=0.0569) followed by Newman-Keuls post hoc test *p<0.05; **p<0.01.

6.3.3.3 Chorea

NBI-675 alone (1 – 7.5 mg/kg) produced a significant induction of chorea at the highest dose (7.5 mg/kg) over the first hour (AUC_{-1-0h}) when compared to vehicle-A treatment (Fig. 6.15 F), however, there was no effect over the 5 hrs ($AUC_{0-5.5h}$) after the L-DOPA/vehicle-B administration, when compared to vehicle-A/B (Fig. 6.15 B – E).

Pre-treatment with NBI-675 had no effect on L-DOPA-induced chorea when compared to L-DOPA alone (Fig. 6.15 B – E), however, the combination L-DOPA/NBI-675 treatment remained significantly different as measured by total ($AUC_{0-5.5h}$) scores (1 & 5 mg/kg) and on-time (5 mg/kg), whereas the peak tended to be increased when compared to NBI-675 alone (Fig. 6.15 B, C & E).

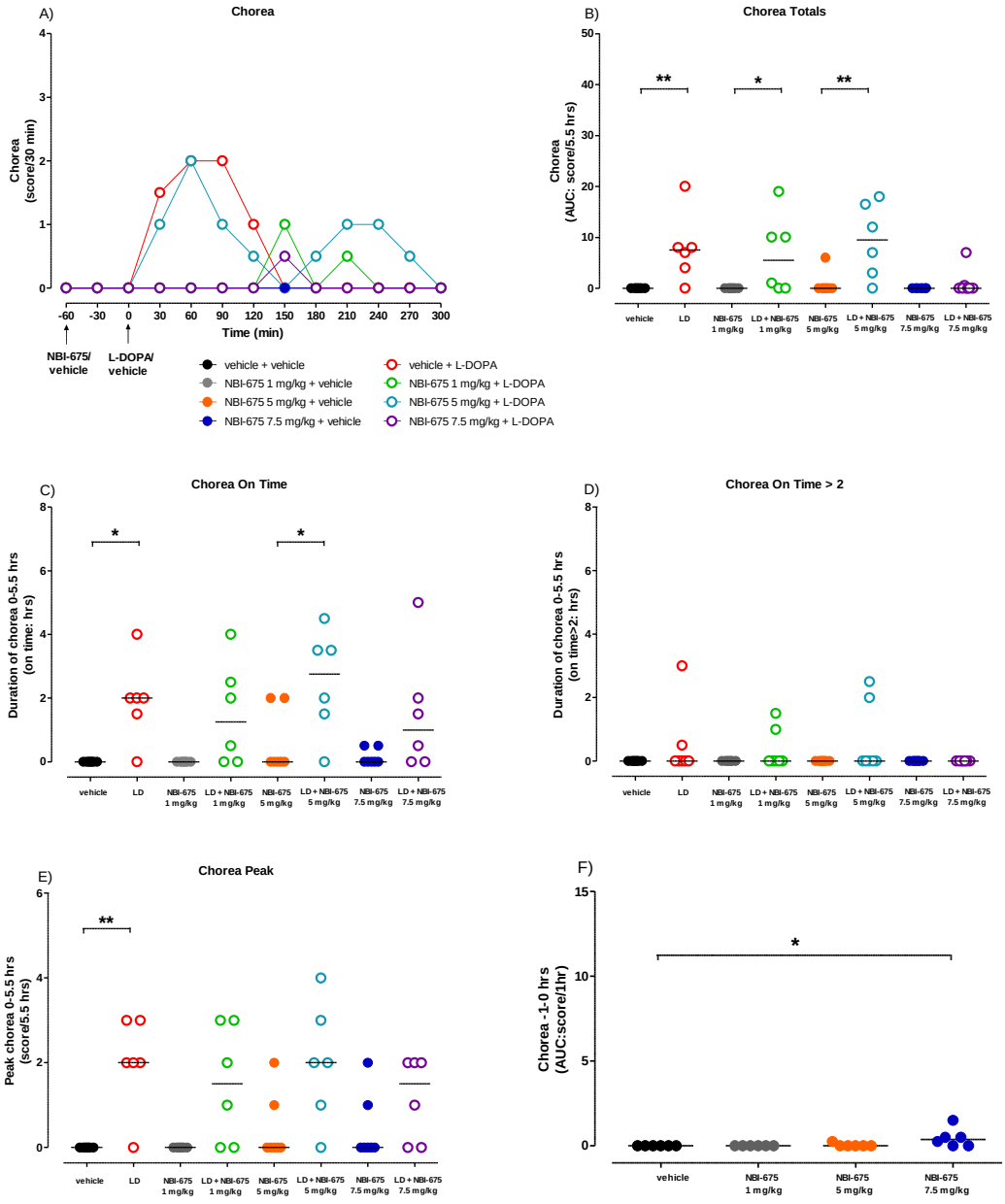


Figure 6.15 Effect of NBI-675 on L-DOPA-induced chorea in MPTP-treated common marmosets

NBI-675 (1 – 7.5 mg/kg p.o.), L-DOPA (8 mg/kg + benserazide 10 mg/kg p.o.) (n=6) A) Chorea time course and B) Total chorea ($AUC_{0\text{-}5.5h}$), C) On-time, D) On-time > 2 and E) Peak chorea after L-DOPA/vehicle-B administration; F) Total chorea in the first hour after NBI-675/vehicle-A ($AUC_{\text{-}1\text{-}0h}$). Data are expressed as time course with median values with error bars omitted for clarity (A) and median with individual counts (B – F). A) No statistical analysis performed; B – F) Data analysed by repeated measures ANOVA; For B, E & F data were transformed $y=\sqrt{y}$; (B) $F=7.530$; Df (7,47); $p<0.0001$; C) $F=5.074$; Df (7,47); $p=0.0005$; D) $F=1.682$; Df (7,47); $p=0.1456$; E) $F=5.067$; Df (7,47); $p=0.0005$; F) $F=5.063$; Df (3,23); $p=0.0128$) followed by Newman-Keuls post hoc test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

6.4 Discussion

The purpose of these studies was to investigate the effects of antagonism of muscarinic receptors with a special emphasis on the muscarinic M4 subtype on the expression of LID and particularly dystonia in MPTP-treated marmosets using clinically utilised non-selective or non-M4 selective anticholinergics. It was hypothesised that selective antagonism of muscarinic M4 receptors with NBI-675 will reduce the expression of L-DOPA-induced dyskinesia and dystonia.

The present study in MPTP-treated marmosets showed that administration of centrally acting non-selective (scopolamine) and with preferential selectivity to M1 receptor (trihexyphenidyl and benztropine) anticholinergics induce dyskinesia with dystonia and chorea. Additionally, these compounds potentiated L-DOPA-induced dyskinesia. In contrast, the novel selective M4 antagonist, NBI-675 alone, showed no significant effect on induction of dyskinesia and dystonia, however, at the highest dose some chorea was expressed. Similarly, there was no effect of NBI-675 on L-DOPA-induced dyskinesia, however, the highest dose of the compound tended to reduce the severity of the dyskinesia. Peripherally acting non-selective methylscopolamine had no effect on induction of dyskinesia, dystonia or chorea when administered alone, but unexpectedly a significant reduction of L-DOPA-induced dyskinesia with dystonia and chorea was observed in this study.

Dyskinesia is the most frequent side effect of long term L-DOPA therapy in PD patients. It is also exhibited in the MPTP-treated primates, once the priming process is established, every subsequent use of L-DOPA or dopamine agonists induces dyskinetic episodes. Therefore, in the current study, initially it was confirmed that animals exhibited a reproducible onset of dyskinesia, manifested by dystonia and chorea, after the administration of L-DOPA alone. This effect was as expected and as previously reported (Pearce *et al.*, 1995; Kuoppamaki *et al.*, 2007). Generally, the severity of dyskinesia reflects the degree of disability and akinesia, where the least disabled animals display milder dyskinesia and this correlates with the degree of nigrostriatal damage (Pearce *et al.*, 1995). This is similar to dyskinesia observed in PD patients, where likelihood of dyskinesia developing in more advanced disease is high and usually is exhibited on the most severely impaired side, thus the likely prevalence and severity of dyskinesia correlates with a degree of striatal denervation (Marconi *et*

al., 1994). Commonly, L-DOPA produces a rapid onset of improvement of motor symptoms which is accompanied by onset of dyskinesia, manifested by dystonia and chorea (Kuoppamaki *et al.*, 2007). This was also observed in this study. Both dystonia and rapid choreic movements were predominantly seen at a peak of L-DOPA duration in MPTP-treated marmosets. Often during the peak-dose dyskinesia, animals appeared akinetic, and were exhibiting dystonia induced by L-DOPA, which was making them less active, and as a result their locomotor activity was lower (Kuoppamaki *et al.*, 2002).

6.4.1 The effect of administration of anticholinergics on expression of dyskinesia in MPTP-treated marmosets

Administration of clinically used centrally acting anticholinergics, scopolamine, trihexyphenidyl and benztropine alone, resulted in almost immediate induction of dyskinesia, consisting of both dystonia and chorea. Previously, it has been reported that anticholinergic treatment induced dyskinesia in parkinsonian patients who were not being treated previously with L-DOPA (Birket-Smith, 1974; Hauser & Olanow, 1993; Linazasoro, 1994). The dyskinesia seen in PD sufferers induced by anticholinergics comprised mainly of oro-buccal form, however, it was also manifested as generalised choreic movements. Withdrawal of anticholinergics resulted in cessation of dyskinesia (Birket-Smith, 1974; Hauser & Olanow, 1993; Linazasoro, 1994). In contrast to the above clinical studies, the manifestation of dyskinesia induced by anticholinergics in the MPTP-treated marmosets was short-lasting and manifested mainly as chorea, with choreic movements of arms and legs, and dystonia, predominantly seen in the trunk and hip. Animals returned to their baseline shortly after the onset. This dyskinetic effect, however, appears to be centrally mediated since peripheral methylscopolamine had no effect on expression of dyskinesia, neither dystonia nor chorea, and it is in agreement with previous studies (Jackson *et al.*, 2014). Interestingly, in contrast to the effect of centrally acting anticholinergics, the highly selective M4 antagonist NBI-675 induced dystonia and chorea only at the highest dose, which was observed after the administration of the compound, but this effect was short lasting.

6.4.2 The effect of pre-treatment with anticholinergics on L-DOPA-induced dyskinesia in MPTP-treated marmosets

Pre-treatment with centrally acting scopolamine and M1 selective trihexyphenidyl significantly potentiated the effect and extended L-DOPA-induced duration of dyskinesia with both manifestation of dystonia and chorea in MPTP-treated marmosets, whereas benztropine showed a tendency to potentiate and extend L-DOPA effect on induction of dyskinesia and dystonia, while significantly extending the duration of L-DOPA-induced chorea. It has been reported in PD patients that the combination of L-DOPA with anticholinergics improve parkinsonian symptoms and reduce dystonia, while often chorea is more pronounced (Poewe *et al.*, 1988). Previous studies in MPTP-treated primates show that "on" dystonia was reduced while "on" chorea was unchanged or often became more marked (Gomez-Mancilla & Bedard, 1993; Jackson *et al.*, 2014). Similarly, in the current study, L-DOPA-induced chorea appeared to be exacerbated after administration of the centrally acting non-M4 selective anticholinergics used in the study. Since the mechanism underlying LID is mediated centrally, peripherally acting methylscopolamine had no effect on LID and both chorea and dystonia, however, interestingly pre-treatment with this drug tended to reduce the L-DOPA effect. This effect is not clear, but since methylscopolamine is a quaternary methylated derivative of scopolamine, has a poor central penetration and high selectivity for both M1 and M3 receptors, therefore the inhibition of peripheral muscarinic receptors might have played a role in this effect, such as the high doses of methylscopolamine could have caused number of side effects.

NBI-675 showed no ability to prevent the expression of L-DOPA-induced dyskinesia, dystonia or chorea, however, there was a trend for it to produce an additive effect on L-DOPA with a tendency of extending the duration of L-DOPA-induced dyskinesia, dystonia and chorea at the lower and mid dose. This tendency of delaying the LID in a manner similar to that seen for locomotor activity and motor disability, as discussed in Chapter 5, could be a possible result of antimuscarinic action in the GI tract, delaying absorption, although this was not confirmed by the PK studies.

Nonetheless, the highest dose showed a tendency of reduction of the L-DOPA effect in some animals. This is unlikely to be an antidyskinetic property, even though all scores for dyskinesia, dystonia or chorea tended to be reduced, as the combination of L-DOPA and the highest dose of NBI-675 were likely to reduce the anti-PD effect

seen with L-DOPA alone (see Chapter 5) on locomotor activity and motor disability, the L-DOPA effect tended to be reduced. Previously, it has been shown that expression of LID correlates closely with locomotor activity, so usually the onset of motor improvement is accompanied by onset of dyskinesia, such that when the same degree of reversal of motor disability is attained with L-DOPA, expression of marked dyskinesia is observed (Kuoppamaki *et al.*, 2007; Lincoln *et al.*, 2016). Comparing the results in Chapter 5, the motor disability correlates with dyskinesia, and the measurements indicate that the highest doses of the drug blunted the L-DOPA effects. This, however, could indicate that the dose of the compound was too high, which possibly could have led to the loss of selectivity and thus reduction of a therapeutic effect, causing the loss of efficacy of the NBI-675 at the higher doses. Consequently, if the selectivity was lost, then the compound could have been acting at other receptors, and this could be the reason for reduced motor activity and inhibited manifestation of dyskinesia as observed. In addition, anticholinergics are known to cause numerous effect in the CNS (Lieberman, 2004), and this could be the reason that animals appeared less active and drowsy, as was speculated in previous chapters (Chapter 3, 4 & 5). Administration of the clinically used centrally acting anticholinergics resulted in an increase of dyskinesia, dystonia and chorea. The effects of anticholinergics on induction of dyskinesia have been previously reported in human studies (Pourcher *et al.*, 1989), and primate studies also indicate that LID and particularly chorea was more pronounced (Gomez-Mancilla & Bedard, 1993; Jackson *et al.*, 2014), therefore this is perhaps not unusual to be seen.

The reason behind the lack of NBI-675 antidyskinetic/antidystonic effect is not entirely clear since it was proposed that muscarinic M4 subtype of receptors, based on their striatal location, could be involved in mediation of dyskinesia without inducing peripheral side effects (Salamone, 1997; Betz *et al.*, 2007). Indeed, studies in rats presented in Chapter 3 showed that NBI-675 reduced involuntary movements in the form of purposeless chewing induced by pilocarpine. Although, one would argue that the rat model is rather simplistic compared to the more complex MPTP-treated marmoset model, where degeneration of nigrostriatal dopaminergic neurons occurs. The rat model, where acute muscarinic response was tested, proved that the cholinergic system indeed is involved in the mediation of involuntary movement. Moreover, it was shown that NBI-675 suppressed the involuntary movements, suggesting a central

action, without inducing the dry mouth, one of the side effect of anticholinergic treatment. Despite that NBI-675 shows high selectivity to M4 receptors, as described in Chapter 1 and 5, it had no effect on LID, and when administered alone, unexpectedly induced short acting mild chorea and dystonia.

Based on this, two different animal models were used in this chapter and Chapter 3. Rat model of pilocarpine-induced purposeless chewing gives acute muscarinic response without any degeneration, whereas MPTP-treated marmoset is more complex giving insights into the human condition PD. The damage to the nigrostriatal tract and loss of striatal DA together with chronic L-DOPA treatment results in altered biochemistry (Chapter 1). In spite that non-selective scopolamine, and preferential M1 selective benztropine and trihexyphenidyl show affinity for M1 receptors, they also show affinity for M4 receptors (Caulfield, 1993), and thus these compounds produced increase in dyskinesia, dystonia and chorea. This may be an M1 receptor effect through the indirect pathway, as it becomes hyperactive due to the loss of D2 inhibition. Blockade of M1 receptors located on D2 MSN may worsen this by further increasing the activity. On the other hand, M4 receptors show inhibitory control of D1 receptor-mediated locomotor stimulation (Gomeza *et al.*, 1999a), due to the opposing effects of the D1 receptor and M4 receptor on adenylyl cyclase activity (Caulfield, 1993; Di Chiara *et al.*, 1994; Onali & Olianas, 2002). Thus, in the MPTP-treated primate, where the indirect pathway is down-regulated due to the loss of dopamine, one would expect that administration of M4 selective compound would further reduce the activity of the direct pathway, so perhaps the likelihood of LID could be reduced. However, this was not the case, and thus may indicate that a balance between M1 and M4 is important for reduction of LID.

In spite of these finding, the exact mechanism of LID and particularly dystonia induced by L-DOPA in PD is still not fully understood. The exact central location of action of anticholinergics is not known, and it is a very complex process, since M1 and M4 receptors are both expressed on striatal projection neurons, and M4 receptors are also expressed on ChI (Ding *et al.*, 2006). The evidence and suggestions coming from the studies described in this thesis and published in the literature implies the importance of both M1 and M4 subtypes of muscarinic receptors (Bonsi *et al.*, 2008; Ding *et al.*, 2011; Erosa-Rivero *et al.*, 2014) as therapeutic targets, therefore perhaps a

development of a mixture of M1/M4 antagonists would be beneficial in the treatment of LID.

Studies described in this and previous chapter are not without limitations. Indeed, the sample size was small, one animal was taken out of the study due to seizures caused by the highest dose of NBI-675. Moreover, two animal groups were used in the study, scopolamine and methylscopolamine was tested in one animal group, and the remaining drugs were tested in another group of animals. This could result in a variation in response. Dosing of L-DOPA 1 hr after the anticholinergic administration, might have made animals anxious or disturbed and they might have appeared to be more agitated, as a result of technicians entering the behaviour room in order to administer L-DOPA orally, thus resulting in an increase of locomotor activity or expression of dyskinesia induced by stress, although this was controlled in the vehicle treated groups.

6.5 Conclusion

L-DOPA-induced dyskinesia is a serious effect of L-DOPA treatment in PD and possibly involves numerous pathways and mechanisms, which still remain unclear. The main aim of this study intended to probe the role of the M4 receptor in the expression of dyskinesia in PD. In contrast to the results obtained in Chapter 3 where the pilocarpine-induced purposeless chewing oral movements in rats were suppressed by the NBI-675, this inhibitory effect was not observed on dyskinesia, dystonia or chorea expressed in the MPTP-treated marmoset. For this reason, the hypothesis of this chapter is rejected. However, since the clinically used anticholinergics tested in this study do not have absolute selectivity for a particular subtype of muscarinic receptor, further studies with more selective compounds or perhaps mixture of muscarinic M1/M4 antagonists would need to be undertaken to elucidate whether this would be a better target for the treatment of this complex manifestation.

Chapter 7 General Discussion

7.1 Summary of results

Anticholinergics are frequently used in the treatment of dystonia and Parkinson's disease either alone or as an adjunct to other forms of therapy. However, their use is often limited due to unwanted side effects, including dry mouth, cognitive disturbance, confusion, blurred vision or urinary retention (Schapira, 2005). There is a considerable evidence that the basal ganglia play a major role in the control of movement, and pathways within the basal ganglia are altered, resulting in the motor symptoms of dystonia and PD. The majority of clinically used anticholinergics are relatively non-selective, and the peripheral side effects are mainly attributable to M1 and M3 receptors. Since the highest expression of M4 subtype of muscarinic receptor is in the striatum, and there is low expression in peripheral tissues, they could be a target for the treatment of the motor dysfunction with minimised side effects profile. For this reason, it was hypothesised that selective antagonists of the muscarinic M4 receptor would control abnormal involuntary movements and motor deficits in dystonia and Parkinson's disease with reduced side effect profile. In order to test this hypothesis, the effect of anticholinergics with differing selectivity for the muscarinic receptor subtypes were investigated in two experimental animal models: pilocarpine-induced chewing rat model of dystonia, and the MPTP-treated common marmoset model of PD. In addition, an indication of peripheral side effects was determined by measuring antagonism of M1/M3-mediated saliva production. The following results were obtained in the individual studies:

1. Selective antagonism of central muscarinic M4 receptors suppressed pilocarpine-induced chewing movements in rats;
2. Selective antagonism of central muscarinic M4 receptors did not induce peripheral side effects, such as oral dryness, in rats;
3. In MPTP-treated common marmoset, selective antagonism of M4 receptors did not show effect in improvement of parkinsonian disability either alone or in combination with L-DOPA;
4. In MPTP-treated common marmoset, selective antagonism of M4 receptors did not improve L-DOPA-induced dyskinesia, but rather extended the duration of dystonia.

Overall, these results support the hypothesis that central cholinergic systems play a role in modulation of involuntary movements, as it has been shown throughout the

studies described in this thesis. Treatment with relatively M4 non-selective anticholinergics resulted in suppression of involuntary movements as presented in a rat model of dystonia described in Chapter 3, and improved motor control in MPTP-treated common marmoset model of PD (Chapter 5 & 6) but with a significant effect on oral dryness (Chapter 4). Similarly, administration of selective muscarinic M4 antagonist NBI-675, suppressed purposeless chewing movements in rats but had no effect on inhibition of pilocarpine-induced salivation, suggesting that selective inhibition of M4 receptors would be useful in the treatment of dystonia but with reduced side effects profile. By contrast, these results do not support the hypothesis that selective M4 antagonists would be beneficial in any aspect of the treatment of PD since the selective NBI-675 showed no improvement of parkinsonian symptoms and motor deficits in MPTP-treated marmosets.

7.2 Specificity of anticholinergics

The selectivity of the currently used anticholinergics in the treatment of both of the disorders plays an enormous role in their efficacy and side effect profile, since the clinically utilized compounds show little selectivities for the specific subtypes of muscarinic receptors, resulting in undesired side effects, which often appear more serious in older patients (Lieberman, 2004). Despite the continuous research, currently there is still lack of highly selective compound that could help to understand the mechanisms beyond these conditions.

Availability of selective subtypes of muscarinic antagonists, and particularly M4 subtypes is limited. In the current study, NBI-675 was chosen as the best available compound showing CNS brain penetration, a logP of 4.6, and high affinity towards M4 receptors (> 900 more selective for M4 compared to M1 subtype, based on the selectivity data provided by Neurocrine Biosciences, Inc.) (Table 1.5). Its rank order towards muscarinic receptors based on affinities is M4>M2>M3>M1>M5 (Neurocrine Biosciences, Inc). Since the compound is in the pre-clinical stage, the available information is very limited. Additionally, there is lack of *in vivo* studies utilizing highly selective muscarinic antagonists in the mediation of motor control. The only compound that has been extensively used to study involvement of M4 subtypes in motor movement is tropicamide, deemed in the literature as having a "moderate" selectivity to M4 receptors (Betz *et al.*, 2007) or called a "purported M4 antagonist"

(Erosa-Rivero *et al.*, 2014), but since its relatively low selectivity to M4 receptor subtypes when compared to M1 (5-fold higher selectivity over M1), it appears incorrect to say that tropicamide is M4 selective.

Relative selectivity of the muscarinic antagonists used in the studies described in this thesis towards particular subtype of muscarinic receptor is mainly based on their pKi values from *in vivo* studies (Section 1.3.2.1), so when tested and used *in vivo* they often may not sufficiently reflect their *in vivo* and clinical relevance. Therefore, the "highly M4 selective" NBI-675 may not be show the same pKi value when tested in the living organism.

Consequently, there is an unmet need for the development of new or improved compounds which would alleviate the symptoms of both diseases without producing unwanted effects.

7.3 Advantages and limitations of the animal models

7.3.1 Pilocarpine-induced purposeless chewing movements in rat

When considering the importance of these results, the advantages and limitations of the techniques used must be considered. Central stimulation of muscarinic cholinergic receptors in rats produces a number of orofacial movements, which has been described in the literature as purposeless chewing or vacuous/tremulous jaw movements (Stewart *et al.*, 1988; Mayorga *et al.*, 1997; Betz *et al.*, 2007) and it share some characteristics of dystonia or PD, and in particular parkinsonian tremor (Stewart *et al.*, 1987; Stewart *et al.*, 1988; Mayorga *et al.*, 1997; Betz *et al.*, 2007). Initially, it was suggested that this behaviour observed in rats reflects tardive dyskinesia, since it can be induced by the administration of DA antagonists or neuroleptics, however, further studies showed that the mechanism differs between the tardive dyskinesia and purposeless chewing movements. The main difference is that tardive dyskinesia is induced by chronic administration of DA antagonists, and they usually worsen it, while it is improved by cholinomimetic drugs. On the contrary, purposeless chewing behaviour is induced by single, acute administration of cholinomimetic drugs and usually anticholinergics improve this effect (Rupniak *et al.*, 1983; Ellison & See, 1989) as was demonstrated in Chapter 3. Moreover, studies revealed that purposeless chewing has higher

frequency than tardive dyskinesia, therefore the former is more indicative of impairment of voluntary movements (Salamone *et al.*, 1998).

Although the model at first appears rather simplistic, as involves mechanical counting of number of chews the rat does, pharmacological studies have shown that this behaviour is related to the ventrolateral striatum, a homologue of ventral putamen in primates, thus indicating its involvement in motor control systems (Salamone *et al.*, 1990). The measurement of the number of purposeless chewing movement may appear to be inconsistent, and that could provide variabilities in findings, nevertheless it has been shown that the measurement can be conducted with a high degree of concordance (Mayorga *et al.*, 1997; Trevitt *et al.*, 1997) and this was the case in the present study which used a rigorous study design with internal controls. The model has been extensively utilised to evaluate the involvement of muscarinic receptors in motor function as described earlier (Stewart *et al.*, 1988; Mayorga *et al.*, 1997; Mayorga *et al.*, 1999; Betz *et al.*, 2007) and in this thesis (Chapter 3). Research conducted with the use of this model have contributed to better understanding of the striatal involvement in generation of involuntary movements (Collins *et al.*, 1991; Cousins *et al.*, 1997; Finn *et al.*, 1997) and work carried out with the used of cholinomimetic, mainly pilocarpine-induced purposeless chewing, have suggested possible involvement of muscarinic M4 subtypes (Betz *et al.*, 2007), which has been confirmed in the current thesis. The model itself is simple and easy to assess, and has presented to meet the validation criteria for using it as a predictive model (Salamone *et al.*, 1998; Cenci *et al.*, 2002). Administration of several muscarinic antagonists on pilocarpine-induced purposeless chewing movement in rats in the current study confirmed previous speculations (Salamone *et al.*, 2001; Betz *et al.*, 2007) and hypothesis that M4 receptors are involved in generation of these movements in rats without inducing unfavourable peripheral side effects.

Despite its limitations the model has demonstrated the cholinergic component involved in involuntary movement, including muscarinic M4 receptors. Whether this will be translated to the treatment of dystonia remains to be seen.

Animal models play an important role in the research into disease states, as they not only give insights into the underlying mechanisms, but also help to improve existing treatment and contribute to explore different avenues for therapeutic development. Many animal models of dystonia have been generated, including genetic rodent

models, drug/toxin-induced rodent models and non-humane primate models. Etiologic models reflect event known that lead to dystonia in humans, such as gene defect or exposure to toxins, whereas phenotypic models reflect a motor syndrome similar to human dystonia which can be seen in animals (Jinnah *et al.*, 2005). Genetic models include spontaneous mutants, transgenic or knockout models. Many models implicate some pathogenic features involved in dystonia, for example involvement of basal ganglia or cerebellum. Transgenic mouse model overexpressing of human mutated torsinA protein is used in the study of DYT1 form of dystonia (Grundmann *et al.*, 2007; Tassone *et al.*, 2011). These transgenic mice usually show impairment in motor learning indicating changes in synaptic plasticity (Martella *et al.*, 2009) and also implicate dopaminergic neurotransmission (Grundmann *et al.,* 2007). The dystonic dt^{sz} hamster, reflects inherited model of paroxysmal dystonia (Jinnah *et al.*, 2008). Dystonic attacks are triggered by stress or sudden movement and can last hours (Loscher *et al.*, 1989). It has been reported that there is altered GABAergic transmission in this model and studies show that dystonic symptoms improve after administration of GABA agonists such as benzodiazepines (Loscher *et al.*, 1989), however, administration of anticholinergics (trihexyphenidyl) did not have effect on severity of dystonia, but delayed onset of attacks (Loscher & Fredow, 1992). This somewhat challenges the validity of the models.

There are also toxin-induced models of dystonia in both rodents and primates. Non-human primate models are used to study focal types of dystonia, e.g. hand dystonia (Byl *et al.*, 1996) or cervical forms induced by GABA agonists and antagonists administration into the GPi or SNr (Burbaud *et al.*, 1998; Klier *et al.*, 2002). They often involve torticollis or abnormal limb postures (Guehl *et al.*, 2009). Administration of 3-nitropropionic acid to both rodents and primates produces lesions of the striatum and manifestations in hindlimbs, trunk, bradykinesia and impaired posture (Fu *et al.*, 1995; Palfi *et al.*, 2000). Since dystonia is considered as a neuronal disorder, there are also models looking at the role of cerebellum in the formation of dystonic movements. Studies have demonstrated that administration of kainic acid, AMPA-glutamate receptor agonist, into the cerebellar vermis in rodents trigger dystonic movement involving limbs and trunk, and are involved in dysfunction of cerebellum (Pizoli *et al.*, 2002; Alvarez-Fischer *et al.*, 2012).

Dystonia has many different aetiologies and is very heterogeneous disorder, therefore generating animal model which would mimic all aspects of the disease is rather unrealistic, nevertheless the most important goal is to use these models to better understand pathophysiology and discover novel therapeutics.

7.3.2 MPTP-treated common marmoset

The non-human primate model is useful in preclinical research as it closely resembles the clinical condition. Systemic administration of MPTP results in degeneration of dopaminergic cells in substantia nigra and closely replicates the motor behaviour seen in man, such as bradykinesia and akinesia, tremor and postural instability, however, the resting tremor is not commonly observed (Jenner, 2009). The major difference between the model and PD is the onset of the neurodegeneration, as in the MPTP-treated primates cell death take place faster than in humans and there is a lack of Lewy bodies (Forno *et al.*, 1988). The neurodegeneration usually closely mimics the later stages of the disease where there is substantial loss of dopaminergic cells, the process is static and non-progressive. Additionally, the disease can be mimicked in relatively young animals, whereas PD in humans is age-dependent (Jenner, 2003b). Similarly, as in PD patients, administration of L-DOPA reverses the parkinsonian symptoms and treatment over period of time results in an expression of dyskinesia that closely resembles seen in man, and includes dystonia, chorea and akathisia (Jenner, 2009). Nevertheless, induction of LID in MPTP-treated animals occurs faster than in humans and as such may involve different biochemical changes (Smith *et al.*, 2003; Kuoppamaki *et al.*, 2007). In spite of these differences, the model has been extensively used in order to find antiparkinsonian treatments. Additionally, many other dopaminergic treatments used in PD are proven to be effective in MPTP-treated marmoset (Close *et al.*, 1990; Jenner, 2008). Despite these benefits, the model has produced failures in translation. Monoamine reuptake inhibitors showed to be effective in reversing motor deficits in MPTP-treated marmosets (Pearce *et al.*, 2002; Hansard *et al.*, 2004), whereas when taken into clinics, they showed no effect in improving parkinsonian disability (Hauser *et al.*, 2007; Rascol *et al.*, 2008).

Currently, there is no ideal non-human primate model for dystonia. However, the MPTP-treated primate model exhibit dystonia as an accompanying feature of PD, as part of LID. It closely resembles the outcome seen in late stage of PD in patients where

dyskinesia develops after commencement of dopaminergic therapy (Jenner, 2008). Similarly, as in the clinics, these involuntary movements can be easily assessed using a semi quantitative rating scales (Langston *et al.*, 2000).

There are several other animal models used to mimic the disease. They involve lesion models, such as 6-OHDA rodent model of motor complications related to the PD treatment. This model results in degeneration of dopaminergic neurons and expresses locomotive, axial, limb and orolingual dyskinesia (Lundblad *et al.*, 2002; Cenci, 2007). Usually extensive neurodegeneration is needed so the animals could develop involuntary movements (Cenci, 2007). The model resembles late-stage disease and does not express "on" and "off" and "end of dose deterioration", as seen in man and MPTP-primates, but only L-DOPA-induced peak dose dyskinesia (Cenci, 2007).

Although, the toxin-induced models of PD have some validity, they show no progression and exhibit rapid-onset of motor complications, most likely because the pathology is mainly restricted to dopaminergic neurons (Cenci, 2007). The process of cell death is thought to reflect those seen in PD, however, poor translation of the potential neuroprotective strategies suggests the need for the better models.

The genetic component underlying PD have led to the development of transgenic models of PD. Despite the attempts to produce some of the models with genetic variations, including mutations of α-synuclein, LRRK, parkin, PINK1, DJ-1 showing some behavioural deficits, many of them failed to produce substantial loss of dopaminergic nigral cells (Duty & Jenner, 2011). Thus, these models also fail to reflect the disease state. In addition, they also represent small proportion of patients making predictions in 'idiopathic' disease difficult.

Despite variability of all models available, it is hard to generate an ideal model, which would mimic all aspects of the diseases. Selection of an appropriate model is often complex, as there are many considerations that should be taken into account, including the convenience, transferability of information, appropriateness, genetic uniformity of organisms, background knowledge, adaptability to experimental manipulation or ethical issues (Tassone *et al.*, 2011).

Two different animal models and two different species were used in the studies described in this thesis. Generally, marmosets brain is bigger than rats, and studies show the differences in the distribution, proportion and size of various neurons in

different structures of BG, indicating fewer and smaller neurons in rats when compared to primates (Hardman *et al.*, 2002). Therefore the differences in relation to BG anatomy and neurochemistry might have had impact on the results.

No model to date has shown to be 100% predictive of effects in man (Blandini & Armentero, 2012). Additionally, there is a lack of models that could be utilised to investigate the cholinergic component that is involved in PD and dystonia, nevertheless the use of cholinergic agonists has been reported to induce involuntary oral movements in rats, resembling chewing, thus this model together with the MPTP-treated primate model provides useful and excellent tool in the assessment of novel treatments with potential antiparkinsonian and antidystonic properties.

Since *in vivo* work gives a better indication of what is happening in the whole system, the caveat of behavioural studies is that the generated data generally show a high degree of variability, and this has also been demonstrated in the current thesis (Chapters 3 – 6) in rats and primate studies. Although, this closely resemble the clinical outcome, where often large variations occur in drug response between patients, it can also indicate necessity of using much larger number of animals in order to achieve significant or desired effect. Many animal models, including non-human primates, are often technically challenging to develop and maintain, as they breed slowly and in a few numbers, and require special animal facilities, therefore it is often not possible to employ bigger sample size, due to the practicality, costs and more importantly, ethical concerns. Nevertheless, the numbers of animals used in the studies in this thesis are comparable to the frequently reported in the literature, where the number of rats was usually 6 – 8 (Mayorga *et al.*, 1999; Betz *et al.*, 2007) and MPTP-treated primates 5 – 8 (Pearce *et al.*, 1998; Kuoppamaki *et al.*, 2002; Kuoppamaki *et al.*, 2007; Jackson *et al.*, 2014).

7.4 Is there a role of muscarinic M4 receptors in control of involuntary movements?

From the behavioural studies utilizing rats and MPTP-treated marmosets reported in this thesis, it was shown that selective muscarinic M4 antagonist NBI-675 suppressed involuntary movement in the form of purposeless chewing movement induced by pilocarpine without induction of peripheral unwanted effects in the form of oral dryness in rats. By contrast, in the marmoset treated with MPTP, as a model of PD and

LID, this compound failed to show antiparkinsonian effect nor improvement of LID. Despite that, both models support the notion that cholinergic system and muscarinic receptors are important targets for the treatment of both dystonia and PD, as other, especially M1 selective anticholinergics showed beneficial effect.

In spite of recent emerging preclinical evidence suggesting M4 involvement in dystonia and Parkinson's disease, the results reported here suggest that the involvement of selective M4 antagonism would be beneficial in control of involuntary movements only in dystonia. NBI-675, as well as M1 (M1/M4) selective pirenzepine resulted in a suppression of involuntary movements induced by pilocarpine in rat studies. However, in the MPTP-treated parkinsonian model, non-selective anticholinergics improved the locomotor symptoms, whereas the selective M4 did not. This suggests the clear difference in the neurochemistry behind dystonia and PD, such that the M4 antagonism may be a suitable pharmacotherapy for the former but not the latter. Indeed, it may be that M1 receptors are better targets for reversal of motor symptoms in PD. Muscarinic M1 subtypes are found on MSN of direct and indirect pathways (Hersch *et al.*, 1994). Since these receptors are excitatory, in a PD state it would indicate that the inhibition of M1 receptors on overactive indirect pathway (loss of D2 inhibition) would oppose the action of lost dopamine and decrease the hyperactivity, triggering stimulation of underactive direct pathway, and this potentially be beneficial.

Inhibitory M4 receptors, are predominantly expressed postsynaptically on direct pathway MSN where they show inhibitory control on D1-mediated locomotor stimulation (Bernard *et al.*, 1992; Gomeza *et al.*, 1999b). Since the direct pathway in PD is underactive, one would expect that inhibition of M4 receptors should improve motor function, however, this was not seen.

Recently, there have been an increasing number of studies investigating the involvement of muscarinic receptor subtypes, and particularly M1 and M4, in locomotion and involuntary movements. For example, a recent study indicated that haloperidol-induced catalepsy was inhibited by an M1 antagonist pirenzepine. However, the difference in affinity of pirenzepine for both M1 and M4 receptors is small (about 5-fold; Table 1.5), therefore authors speculate that this effect could be contributed to a blockade of M1/M4 muscarinic receptors (Erosa-Rivero *et al.*, 2014).

Previously it has been shown that D1-M4-KO mutant mice exhibited reduction in haloperidol-induced catalepsy, indicating involvement of M4 subtypes in mediation of motor control (Jeon *et al.*, 2010).

Moreover, a recent study by Ztaou et al., (2016) demonstrated that muscarinic M1 and M4 receptors are both involved in regulation of motor function. In this study, the 6-OHDA mice model of PD was utilised together with the photoinhibition of ChI, mutant mice lacking M4 receptors and preferential blockade of M1 and M4 receptors. Despite the fact that telenzepine and tropicamide are not highly selective for muscarinic M1 and M4 receptors, respectively, the study suggested that both of these compounds were able to relieve the motor deficits produced by 6-OHDA. Most likely, this effect was due to the inhibition of the postsynaptic M1 receptors located on MSN indirect pathway by telenzepine, while antiparkinsonian effect of tropicamide was eliminated in lesioned mutant mice suggesting involvement of postsynaptic M4 receptors located on MSN direct pathway (Ztaou *et al.*, 2016). These studies support the notion that both of these receptors may play a role in motor control, and perhaps in mediation of involuntary movements.

In another study, muscarinic M4 receptor deficient mice demonstrated increase in basal locomotor activity. Administration of D1 centrally acting agonist, SKF38393, resulted in an increase of enhancement of locomotor activity (Gomeza *et al.*, 1999b), which supports the idea that M4 receptors located on the direct pathway MSN show inhibitory effect on D1 receptor-stimulated locomotor activity. This could perhaps indicate that multiple receptor subtypes play a role in motor control.

In the current study in this thesis, administration of non-M4-highly-selective anticholinergics, including trihexyphenidyl, benztropine and scopolamine (Table 1.5), evoked better response as such the improvement of parkinsonian symptoms and increase in locomotion, than that seen with NBI-675, suggesting that M1 or a mixture of M1/M4 receptor could be a potential target for symptomatic treatment of PD.

Growing evidence coming from animal studies suggests involvement of M1 receptors in DYT1 form of dystonia. The electrophysiological studies using knock-in of torsinA gene mouse model of DYT1 dystonia show changes in MSN, corticostriatal synaptic plasticity and irregular reaction of ChI resulted from activation of D2 receptors (Martella *et al.*, 2014). The high-frequency stimulation of MSN did not cause long-

term depression (LTD) but enhancement of long-term potentiation (LTP). Additionally, blockade of M1 receptors by pirenzepine and trihexyphenidyl on the corticostriatal slices resulted in a restoration of LTD (Martella *et al.*, 2014).

Interestingly, a recent series of experiments conducted by Shen et al., (2015) showed that M4 positive allosteric modulator (PAM) administered with L-DOPA, reduced dyskinesia in a mouse unilateral model of 6-OHDA and non-human primate MPTP model of PD and the effect of reduction of LID was similar to that seen by amantadine. It was demonstrated that increase in M4 signalling by PAM blunts D1 receptor-mediated LTP in models of LID, by lessening induction of LTP and allowing depotentiation (Shen *et al.*, 2015). This suggests that both subtypes of muscarinic receptors may be involved in the plastic changes in basal ganglia that might result in abnormal movement, however, more studies need to be conducted to clarify these findings.

Taken together data presented in this thesis, particularly rat studies (Chapter 3), where both M1 and M4 antagonists showed high efficacy in suppression of pilocarpine-induced purposeless chewing movements, suggest that a development of mixed M1/M4 antagonist would provide aid with reduced side effect profile. Additional benefit would bring compounds that alleviate the LID without reducing L-DOPA therapeutic efficacy, as this is a major challenge, since many drugs reduce dyskinesia but do not improve parkinsonian symptoms. Nonetheless, a major limitation of this approach are adverse effects related to muscarinic M1 receptor, both central and peripheral, such as cognitive impairment and dementia, sedation, confusion or dry mouth and eyes (Lampela *et al.*, 2015). Despite that, further studies with more appropriate compounds need to be conducted to clarify these issues.

7.5 Other possible treatments

7.5.1 Nicotinic acetylcholine receptors

Acetylcholine exerts also its effect through nicotinic receptors and perhaps these receptors could also be a target for the treatment (Wess *et al.*, 2007). As described earlier (Chapter section 1.3.1), nicotinic receptors are expressed in the striatum at glutamatergic terminals and when activated, they release glutamate from corticostriatal terminals (Marchi *et al.*, 2002; Marchi & Grilli, 2010). However, there

is less indication for a role of the nicotinic receptors. Historically nicotine has been used to treat writer's cramp and spastic dystonia though local or transdermal application (Vaughan *et al.*, 1997; Murase *et al.*, 2000). More recently it has been shown that mutations in α4 or β2 nicotinic receptor subunits can contribute to a dystonic arousal state (Teper *et al.*, 2007), however, to date there is little evidence for a role of nicotinic antagonists in the treatment of dystonia.

By contrast, in PD there is an historic link to therapy related to the nicotinic receptor. It has been demonstrated that smokers have reduced risk of PD, which is associated with the duration and strength of smoking (Thacker *et al.*, 2007), although this may be a disease modifying effect. Interestingly, reduced motor complications have been linked to striatal α7 nACh receptors, suggesting the potential of this receptor to manage motor complications in PD (Morissette *et al.*, 2016). Using ACh knock out mice, Quik *et al*., (2013) have suggested a role for α6β2*, α4β2* and α7 nAChRs in L-DOPA-induced AIMs, thus proposing future targets for reducing LID in Parkinson's disease (Quik *et al.*, 2013). Indeed, α7 nicotinic receptor agonists ABT-126 and ABT-107 reduce LID in a non-human primate model of PD (Zhang *et al.*, 2014; Zhang *et al.*, 2015), although the α7 nicotinic agonist AQW051 failed to show benefit on PD symptoms or LID (Trenkwalder *et al.*, 2016). Further work is needed to fully elucidate the role of the nicotinic receptors in motor function.

7.5.2 Glutamate receptors

Another approach could be to target other neurotransmitters in the basal ganglia. Glutamate is one of the major neurotransmitters in the BG, and plays an important role in the control of movement, thus is a target for modification in the control of motor function.

In the DYT1 mouse models of torsion dystonia there is evidence of altered glutamatergic activity in the cerebellum but not in the striatum (Vanni *et al.*, 2015), however, functional alterations in the basal ganglia circuits have been reported in both DYT1 dystonia patients and rodent models (Yokoi *et al.*, 2015), but no reported changes in levels of striatal ionotropic glutamate receptor subunits or, interestingly the muscarinic M4 receptor and adenosine A2a receptor. However, the story may be more complicated as Sciamanna *et al.*, (2014) showed that negative modulation of mGlu5 receptors with dipraglurant may counteract abnormal D2 receptor responses,

normalizing cholinergic cell excitability in the Tor1a(+/ΔGAG) mice and transgenic mice overexpressing human torsinA (hMT1) (Sciamanna *et al.*, 2014). Clearly considerably more work needs to be done to fully understand the role of glutamate in dystonia.

There is considerably more understanding of the role of glutamate in PD. The alterations in direct and indirect pathway involve glutamatergic hyperactivity, causing the motor symptoms of PD and contributing to the pathophysiology of dyskinesia.

Amantadine, a weak NMDA receptor antagonist, is the only clinically used drug to treat dyskinesia (Hallett *et al.*, 2006). However, its non-selective pharmacology makes it intolerable for some patients. There have been a number of attempt to find better treatments, and recently a new formulation of amantadine, ADS-5102 has shown to reduce LID when compared to placebo-treated patients in phase III study. Moreover, it has been reported that "on" time was increased by >2 hrs and "off" time was reduced by 0.9 hrs compared to placebo. A number of patients still experienced side effects, including hallucinations, peripheral oedema, dizziness, dry mouth, constipation, falls, anxiety, confusion, livedo reticularis, abnormal dreams, depression and headaches (Pahwa *et al.*, 2015).

Of particular interest in the treatment of PD are metabotropic glutamate receptors. They are G-protein coupled receptors (GPCRs) and classified into three groups I, II and III. The group III are presynaptically located and coupled to $G_{o/i}$ G proteins (Johnson *et al.*, 2009) and mGlus 4, 7 and 8, which belong to this group, are expressed in the BG where play a role in control of motor function (Niswender *et al.*, 2008). Activation of these receptors decreases GABA transmission in the striato-pallidal synapse (Conn *et al.*, 2009).

In MPTP-treated macaque model of LID, the mGlu5 NAM, dipraglurant, reduced dyskinesia, including dystonia and chorea, but had no effect on L-DOPA efficacy (Bezard *et al.*, 2014), the latter often being seen with drugs that reduce the severity of dyskinesia.

Studies on 6-OHDA-lesioned rats show that group III mGlu agonists reversed akinesia when injected bilaterally into the globus pallidus and reduced haloperidol-induced catalepsy in the rat model of PD (Lopez *et al.*, 2007). Another mGlu4 agonist, L-AP4 showed antiparkinsonian properties in both haloperidol-induced catalepsy and reserpine-model of PD, and studies in mGluR4 knock out mice demonstrated reduction

of striato-pallido synapse transmission, as it is believed that antiparkinsonian effects are thought to be a result of suppression of the increased activity in the indirect pathway (Valenti *et al.*, 2003). Since highly conserved orthosteric side of the receptor make it difficult to develop highly selective compounds, development of positive allosteric modulators appears to be attractive (Niswender *et al.*, 2008).

In *in vitro* studies it is suggested that GluR Group III agonists control nigral glutamate release. This antiparkinsonian potential is extended to *in vivo* studies where direct injection into the SN reversed reserpine-induced akinesia (Austin *et al.*, 2010). Lu AF21934, a selective mGlu4 PAM, showed positive antiparkinsonian effect by alleviating haloperidol-induced catalepsy, and, when combined with L-DOPA following 6-OHDA lesions, decreased incidence of LID indicating antiparkinsonian and antidyskinetic properties (Bennouar *et al.*, 2013).

Dipraglurant the mGluR5 NAM reduces LID in non-human primates, and in clinical studies was found to reduce peak dose dyskinesia and increase "on" time (Bezard *et al.*, 2014; Tison *et al.*, 2016). Similarly, mGluR5 antagonists also showed to have antidyskinetic properties in PD patients with moderate to severe LID. Clinical study has shown that mavoglurant (AFQ056) reduced severity of dyskinesia (Berg *et al.*, 2011).

Clearly there has been a lot of interest in targeting glutamate receptors, and in PD at least there has been some positive translation to clinic.

7.5.3 Serotonergic system

A number of studies performed in rodents implicate changes to the serotonin system in the pathology of the dystonia (Ledoux *et al.*, 1994) but as yet no preclinical studies have suggested a pharmacological target.

Clinical studies have investigated the effect of serotonin in dystonia, but most of these have been based on individual case reports. For instance, aripiprazole, an atypical antipsychotic with partial agonist activity at the D2 and 5-HT_{1A} receptors and an antagonist at 5-HT_{2A} receptors may be more likely to cause movement disorders than other atypical antipsychotics, suggesting a role for 5-HT receptors. Recently Smit *et al.*, (2016) performed a systematic review of the literature and suggested that there was an association between dystonia and the serotonergic system (Smit *et al.*, 2016). However, there was no suggestion of any particular serotonin receptor target.

Serotonergic system plays an important role in pathogenesis of LID as release of dopamine from serotonergic neurons can contribute to pulsatile stimulation of DA receptors triggering appearance of dyskinesia. Indeed, studies on 6-OHDA-lesioned rats primed with L-DOPA have demonstrated that 5-HT_{1A} receptor agonist 8-OH-DPAT attenuates L-DOPA-induced dyskinesia (Dupre *et al.*, 2008; Bishop *et al.*, 2009). This antidyskinetic effect of various 5-HT_{1A} receptor agonists was also seen in MPTP-treated monkeys (Bibbiani *et al.*, 2001; Grégoire *et al.*, 2009). The mixed 5-$HT_{1A/1B}$ receptor agonist, eltoprazine, showed antidyskinetic effect in 6-OHDA-lesioned rats and MPTP-treated macaques, however, reduction of L-DOPA antiparkinsonian effect was also observed (Bezard *et al.*, 2013). Similarly, clinical study demonstrated that a single dose of eltoprazine reduces L-DOPA-induced dyskinesia and did not alter antiparkinsonian effects of L-DOPA (Svenningsson *et al.*, 2015). Other clinical studies also reported antidyskinetic effect of 5-HT_{1A} agonists on LID (Bara-Jimenez *et al.*, 2005; Politis *et al.*, 2014) indicating that selective serotonin 5-HT_{1A} agonists show promise in alleviating L-DOPA-induced dyskinesia.

7.6 Final conclusion

Overall the work described in this thesis supports the continued examination of the involvement of muscarinic receptors subtypes in the formation of involuntary movements. Although, these studies do not represent ways of defining the causal factors underlying the changes that appear in BG circuits that contribute to dystonia, nor the neural degeneration seen in PD, they focused on the observational responses of drug treatment in predictive animal models of PD and dystonia to measure specific outcomes of drug-induced behaviour, such as motor disability and involuntary movements (dystonia). This is the first time that studies with the use of selective muscarinic M4 antagonist have been carried out in the MPTP-treated marmoset model of PD and LID. While the results reported in this work have partly failed to support the hypothesis of this thesis, due to the rather unexpected results from MPTP-treated marmoset studies, the findings nevertheless emphasise the importance of the cholinergic system and selective inhibition of M1/M4 subtypes of muscarinic receptors and their implication in both disorders. The exact mechanisms underlying both idiopathic and levodopa-induced dystonia as well as dyskinesia are still not fully understood despite the ongoing research, but with our expanding knowledge, further

treatment strategies with the use of different models or other targets may provide necessary interventions to reduce these condition in the future.

Chapter 8 References

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